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(71) Applicant (for all designated States except US): THE AMERICAN NATIONAL RED CROSS [US/US]; 8111 Gatehouse Road, Falls Church, VA 22042 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): SAENKO, Evgueni,

L. [RU/US]; 9617 Horizon Run Road, Gaithersburg, MD 20879 (US). STRICKLAND, Dudley, K. [US/US]; 4124 Barnsley Lane, Olney, MD 20832 (US).

(74) Agents: GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

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(54) Title: METHODS OF REDUCING FACTOR VIII CLEARANCE AND COMPOSITIONS THEREFOR

(57) Abstract: The present invention provides methods of increasing the half-life of factor VIII. More specifically, the invention provides methods of increasing the half-life of factor VIII by substituting amino acids in the A2 domain or in the C2 domain of factor VIII or in both domains. It further provides factor VIII mutants produced by these methods. The invention also provides a method of using receptor-associated protein (RAP) to increase the half-life of factor VIII. The invention also provides polynucleotides encoding the mutant factor VIII, polynucleotides encoding RAP, and methods of treating hemophilia using the polypeptides and polynucleotides of the invention.

Methods of Reducing Factor VIII
Clearance and Compositions Therefor

Background of the Invention

Field of the Invention

10   This invention relates generally to a mutant factor VIII having increased

half-life, methods of production, pharmaceutically acceptable compositions and

uses thereof. This invention also relates to a method of using receptor associated

protein to increase the half-life of factor VIII, methods of production,

pharmaceutically acceptable compositions and uses thereof.

Related Art

15   Coagulation of blood occurs by either the "intrinsic pathway" or the

"extrinsic pathway," whereby certain blood proteins interact in a cascade of

proteolytic activations to ultimately convert soluble fibrinogen to insoluble fibrin.

These threads of fibrin are cross-linked to form the scaffolding of a clot; without

20   fibrin formation, coagulation cannot occur.

The intrinsic pathway consists of seven steps: (1) the proteolytic activation

of factor XII; (2) activated factor XII cleaves factor XI to activate it; (3) activated

factor XI cleaves factor IX, thereby activating it; (4) activated factor IX interacts

with activated factor VIII to cleave and activate factor X; (5) activated factor X

25   binds to activated factor V on a membrane surface, which complex proteolytically

cleaves prothrombin to form thrombin; (6) thrombin proteolytically cleaves

fibrinogen to form fibrin; (7) fibrin monomers assemble into fibrils, which are

then cross-linked by factor XIII.

The extrinsic pathway consists of the following steps: (1) upon rupture of a blood vessel, factor VII binds to tissue factor, a lipoprotein present in tissues outside the vascular system; (2) factor VII is activated to factor VIIa by proteolytic cleavage; and (3) the factor VIIa-tissue factor complex cleaves and activates factor X. Thereafter, the extrinsic pathway is identical to the intrinsic pathway, *i.e.* the two pathways share the last three steps described above.

The plasma glycoprotein factor VIII circulates as an inactive precursor in blood, bound tightly and non-covalently to von Willebrand factor. Factor VIII (fVIII) is proteolytically activated by thrombin or factor Xa, which dissociates it from von Willebrand factor (vWf) and activates its procoagulant function in the cascade. In its active form, factor VIIIa (fVIIIa) functions as a cofactor for the factor X activation enzyme complex in the intrinsic pathway of blood coagulation, and it is decreased or nonfunctional in patients with hemophilia A.

In hemophilia, blood coagulation is impaired by a deficiency in certain plasma blood coagulation factors. People with deficiencies in factor VIII or with antibodies against factor VIII suffer uncontrolled internal bleeding that may cause a range of serious symptoms unless they are treated with factor VIII. Symptoms range from inflammatory reactions in joints to early death. The classic definition of factor VIII, in fact, is that substance present in normal blood plasma that corrects the clotting defect in plasma derived from individuals with hemophilia A. A deficiency in vWf can also cause phenotypic hemophilia A because vWf is an essential component of functional factor VIII. In these cases, the half-life of factor VIII is decreased to such an extent that it can no longer perform its particular functions in blood-clotting.

The fVIII protein consists of a homologous A and C domains and a unique B domain which are arranged in the order Al-A2-B-A3-Cl-C2 (Vehar, G.A., *et al.*, *Nature* *312*:337-340 (1984)). It is processed to a series of Me^{2+} linked heterodimers produced by cleavage at the B-A3 junction (Fay, P.J., *et al.*, *Biochem. Biophys. Acta.* *871*:268-278 (1986)), generating a light chain (LCh)

consisting of an acidic region (AR) and A3, C1, and C2 domains and a heavy chain (HCh) which consists of the A1, A2, and B domains (Fig. 1).

Activation of fVIII by thrombin leads to dissociation of activated fVIII (fVIIIa) from vWf and at least a 100-fold increase of the cofactor activity. The fVIIIa is a A1/A2/A3-C1-C2 heterotrimer (Fay, P.J., *et al., J. Biol. Chem* 266:8957-8962 (1991)) in which domains A1 and A3 retain the metal ion linkage (Fig. 1) and the stable dimer A1/A3-C1-C2 is weakly associated with the A2 subunit through electrostatic forces (Fay, P.J., *et al., J. Biol. Chem* 266:8957-8962 (1991)). Spontaneous dissociation of the A2 subunit from the heterotrimer results in non-proteolytic inactivation of fVIIIa.

Infusion of fVIII/vWf complex or purified plasma or recombinant fVIII into patients with severe hemophilia A who do not have fVIII (Fijnvandraat, K., *et al., Thromb. Haemostas.* 77:298-302 (1997); Morfini, M., *et al., Thromb. Haemostas.* 68:433-435 (1992)) or in normal individuals (Over, J., *et al., J. Clin. Invest.* 62:223-234 (1978)) results in a similar fVIII disappearance with a half-life of 12-14 hours. Although the complex between fVIII and vWf is crucial for normal half-life and level of factor VIII in the circulation, the mechanisms associated with turnover of fVIII/vWf complex are not well defined.

The human factor VIII gene was isolated and expressed in mammalian cells (Toole, J. J., *et al., Nature* 312:342-347 (1984); Gitschier, J., *et al., Nature* 312:326-330 (1984); Wood, W. I., *et al., Nature* 312:330-337 (1984); Vehar, G. A., *et al., Nature* 312:337-342 (1984); WO 87/04187; WO 88/08035; WO 88/03558; U.S. Pat. No. 4,757,006), and the amino acid sequence was deduced from cDNA. Capon *et al.,* U.S. Pat. No. 4,965,199, disclose a recombinant DNA method for producing factor VIII in mammalian host cells and purification of human factor VIII. Human factor VIII expression in CHO (Chinese hamster ovary) cells and BHKC (baby hamster kidney cells) has been reported. Human factor VIII has been modified to delete part or all of the B domain (U.S. Pat. No. 4,868,112), and replacement of the human factor VIII B domain with the human factor V B domain has been attempted (U.S. Pat. No. 5,004,803). The cDNA

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sequence encoding human factor VIII and predicted amino acid sequence are shown in SEQ ID NOs:1 and 2, respectively.

U.S. Patent No. 5,859,204, Lollar, J.S., describes mutants of human factor VIII having reduced antigenicity and reduced immunoreactivity.

Porcine factor VIII has been isolated and purified from plasma (Fass, D. N., et al., *Blood 59*:594 (1982)). Partial amino acid sequence of porcine factor VIII corresponding to portions of the N-terminal light chain sequence having homology to ceruloplasmin and coagulation factor V and largely incorrectly located were described by Church, et al., *Proc. Natl. Acad. Sci. USA 81*:6934 (1984). Toole, J. J., et al., *Nature 312*:342-347 (1984) described the partial sequencing of the N-terminal end of four amino acid fragments of porcine factor VIII but did not characterize the fragments as to their positions in the factor VIII molecule. The amino acid sequence of the B and part of the A2 domains of porcine factor VIII were reported by Toole, J. J., et al., *Proc. Natl. Acad. Sci. USA 83*:5939-5942 (1986). The cDNA sequence encoding the complete A2 domain of porcine factor VIII and predicted amino acid sequence and hybrid human/porcine factor VIII having substitutions of all domains, all subunits, and specific amino acid sequences were disclosed in U.S. Pat. No. 5,364,771 by Lollar and Runge, and in WO 93/20093. More recently, the nucleotide and corresponding amino acid sequences of the A1 and A2 domains of porcine factor VIII and a chimeric factor VIII with porcine A1 and/or A2 domains substituted for the corresponding human domains were reported in WO 94/11503. U.S. Patent No. 5,859,204, Lollar, J.S., discloses the porcine cDNA and deduced amino acid sequences.

Cellular endocytosis mediated by LRP was shown to be a mechanism of removal of a number of structurally unrelated ligands including several proteins related to coagulation or fibrilolysis. These ligands are: complexes of thrombin with antithrombin III (ATIII), heparin cofactor II (HC11) (Kounnas, M.Z., et al., *J. Biol. Chem. 271*:6523-6529 (1996)), protease nexin I (Knauer, M.F., et al., *J. Biol. Chem. 272*:12261-12264 (1997)), complexes of urokinase-type and tissue-

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type plasminogen activators (u-PA and t-PA, respectively) with plasminogen activator inhibitor (PAI-1) (Nykjaer, A., *et al.*, *J. Biol. Chem.* *267*:14543-14546 (1992); Orth, K., *et al.*, *Proc. Natl. Acad. Sci.* *89*:7422-7426 (1992)), thrombospondin (Mikhailenko, I., *et al.*, *J. Biol. Chem.* *272*:6784-6791 (1997)), tissue factor pathway inhibitor (TFPI) (Warshawsky, I., *et al.*, *Proc. Natl. Acad. Sci.* *91*:6664-6668 (1994)), and factor Xa (Narita, M., *et al.*, *Blood 91*:555-560 (1998); Ho, G., *et al.*, *J. Biol. Chem 271*:9497-9502 (1996)).

LRP, a large cell-surface glycoprotein identical to α_2-macroglobulin receptor (Strickland, D.K., *et al.*, *J. Biol. Chem.* 265:17401-17404 (1990)), is a member of the low density lipoprotein (LDL) receptor family which also includes the LDL receptor, very low density lipoprotein (VLDL) receptor, vitellogenin receptor and glycoprotein 330 receptor. LRP receptor consists of the non-covalently linked 515 kDa α-chain (Herz, J., *et al.*, *EMBO J.* 7:4119-4127 (1988)) containing binding sites for LRP ligands, and the 85 kDa transmembrane β-chain. Within the α-chain, cluster of cysteine-rich class A repeats is responsible for ligand binding (Moestrup, S. K., *et al.*, *J. Biol. Chem 268*:13691-13696 (1993)). In contrast to the acidic ligand binding region in LRP, ligands of LRP expose regions rich in positively charged amino acid residues (Moestrup, S.K., *Biochim. Biophys. Acta 1197*:197-213 (1994)). This type of binding and 31 class A repeats present in LRP may be responsible for its wide ligand diversity and ability to serve as a multi-ligand clearance receptor. LRP is expressed in many cell types and tissues including placenta, lung and brain (Moestrup, S.K., *et al.*, *Cell Tissue Res.* *269*:375-382 (1992)) and is a major endocytic receptor in the liver (Strickland, D.K., *et al.*, *FASEB J.* *9*:890-898 (1995)).

A 39 kDa receptor-associated protein (RAP) binds to LRP with high affinity (K_d=4 nM (27)) and inhibits binding and LRP-mediated internalization and degradation of all ligands (Moestrup, S.K., *Biochim. Biophys. Acta* 1197:197-213 (1994); Williams, S.E., *et al.*, *J. Biol. Chem.* *267*:9035-9040 (1992)), therefore serving as a useful tool for testing whether LRP is involved in endocytosis of a given ligand.

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Severe hemophiliacs, who number about 10,000 in the United States, can be treated with infusion of human factor VIII, vWf/factor VIII complex or vWf which will restore the blood's normal clotting ability if administered with sufficient frequency and concentration. However, supplies have been inadequate and problems in therapeutic use occur due to difficulty in isolation and purification, immunogenicity, and the necessity of removing the AIDS and hepatitis infectivity risk.

Several preparations of human plasma-derived factor VIII of varying degrees of purity are available commercially for the treatment of hemophilia A. These include a partially-purified factor VIII derived from the pooled blood of many donors that is heat- and detergent-treated for viruses but contains a significant level of antigenic proteins; a monoclonal antibody-purified factor VIII that has lower levels of antigenic impurities and viral contamination; and recombinant human factor VIII, clinical trials for which are underway. Unfortunately, human factor VIII is unstable at physiologic concentrations and pH, is present in blood at an extremely low concentration (0.2 µg/ml plasma), and has low specific clotting activity.

The problems associated with the commonly used, commercially available, plasma-derived factor VIII have stimulated significant interest in the development of a better factor VIII product. There is a need for a more potent factor VIII; a factor VIII that is stable at a selected pH and physiologic concentration; a factor VIII that is has a longer half-life in circulating blood.

Summary of the Invention

The present invention relates to a method of increasing the half-life of factor VIII. More specifically, the present invention relates to a mutant of factor VIII having reduced clearance from plasma.

In one embodiment, the mutant factor VIII has one or more amino acid substitutions in the A2 domain.

In a preferred embodiment, the substituted amino acid(s) are important for receptor-dependent clearance of factor VIII, such that the resulting mutant factor VIII has a longer (increased) circulating half-life.

In another embodiment, the mutant factor VIII has one or more amino acid substitutions in the C2 domain.

In a preferred embodiment, the substituted amino acid(s) are important for receptor-independent clearance of factor VIII, such that the resulting mutant factor VIII has a longer (increased) circulating half-life.

In yet another preferred embodiment, amino acid(s) important for receptor-dependent clearance in the A2 domain and amino acid(s) important for receptor-independent clearance in the C2 domain are substituted, such that the resulting mutant factor VIII has an increased circulating half-life.

The invention also relates to a method of using receptor associated protein (RAP) to increase the half-life of factor VIII. Further aspects of the invention include a method of producing factor VIII mutants having an increased half-life, pharmaceutically acceptable compositions thereof, and a method of treating factor VIII deficiency using mutant factor VIII of the invention and/or RAP.

Brief Description of the Figures

FIG. 1. Domain structure of fVIII and its fragments. The domain structure of mature fVIII protein is shown in line 1. The LCh acidic region is labeled as AR. Thrombin-cleaved LCh (A3-C1-C2), heterotrimeric fVIIIa (A1/A2/3-C1-C2) and heterodimer A1/A3-C1-C2 are shown in lines 2, 3 and 4.

FIGs. 2A and 2B. The amino acid sequence of mature, B-domainless fVIII (SEQ ID NO:5; composed from GenBank Accession No. X01179). The A2 sequence within fVIII is underlined and the sequence of the LRP binding site (residues 484-509) within A2 is indicated with asterisks. The amino acid residues shown as one-letter amino acid abbreviations.

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FIGS. 3A and 3B. The deduced amino acid sequence of full-length factor VIII (SEQ ID NO:2; from GenPep Accession No. CAA25619.1 and GenBank Accession No. X01179).

FIG. 4. The deduced amino acid sequence of RAP (SEQ ID NO:4; GenBank Accession No. M63959). The signal sequence (amino acids 1-34) is underlined and the LDL receptor binding region (amino acids 237-353) is indicated with asterisks.

FIGS. 5A and 5B. Binding of ^{125}I-fVIII to purified LRP by ligand competition assay. ^{125}I-fVIII (1 nM) was incubated for 1 h at 37°C in wells coated with LRP (●) or BSA (○) in the presence of increasing concentrations of unlabeled competitors, fVIII (●, ○) or vWf (Δ), *panel A*, and RAP (●, ○), *panel B*. In the experiment (Δ), ^{125}I-fVIII was preincubated with vWf for 30 min at 37°C, prior to its addition to the wells. Following incubation, the wells were washed and ^{125}I-fVIII binding was determined. Binding of ^{125}I-fVIII in the presence of unlabeled fVIII, vWf, or RAP is expressed as the percentage of ^{125}fVIII binding, when no competitor was added. Each point represents the mean value of triplicates and the error bars display the standard deviation. The curves show a best fit of the data to a model describing heterologous ligand displacement from a single class of binding sites using the program LIGAND.

FIG. 6. Effect of fragments of fVIII on its binding to LRP. ^{125}fVIII (1 nM) and increasing concentrations of unlabeled HCh (●), A2 (▲), LCh (○) or A1/A3-C1-C2 (Δ) were incubated with LRP as described in Fig. 5. Each point represents the mean value and the standard deviation of the triplicates. The data were fitted as in Fig. 5 to a model describing heterologous ligand displacement from a single class of binding sites with K_i values of 120 and 132 nM for HCh and A2, respectively.

FIGS. 7A and 7B. Effect of monoclonal antibodies and synthetic peptides on ^{125}fVIII binding to purified LRP. *Panel A*, ^{125}fVIII (1 nM) and increasing concentrations of mAbs 413 (●) or T5 (○) were added to LRP coated wells as described in Fig. 5. In the control experiment (Δ), ^{125}If-VIII and increasing

concentrations of mAb 413 were added to BSA coated wells. *Panel B*, ^{125}I-fVIII and increasing concentrations of synthetic peptides consisting of the A2 domain residues 484-509 (●) or 432-456 (○) were added to LRP coated wells. In the control experiment (Δ), ^{125}I-fVIII and increasing concentrations of the peptide 484-509 were added to BSA coated wells. In the panels A and B, binding of ^{125}I-fVIII in the presence antibodies or peptides is expressed as the percentage of its binding, when no competitor was added. The mean and standard deviation of the triplicate measurements are presented.

FIGS. 8A and 8B. Internalization and degradation of ^{125}I-fVIII/vWf complex by LRP-expressing (MEF) and LRP-deficient (PEA 13) fibroblasts. Wells containing 2x10 of each MEF (○, ●) or PEA 13 cells (Δ, ▲) were incubated with 1 nM ^{125}I-fVIII/vWf in the absence (closed symbols) or presence (opened symbols) of RAP (1 μM). ^{125}I-fVIII/vWf complex was prepared by incubation of ^{125}I-fVIII with unlabeled vWf at a molar ratio 1:50 for 30 min at 37°C. At the indicated times, the amounts of internalized ^{125}I-fVIII (*panel A*) and degraded ^{125}I-fVIII (*panel B*) by the MEF and PEA 13 fibroblasts were determined as described under Experimental Procedures. In the experiment (∇), degradation of ^{125}I-fVIII (1 nM) by MEF cells in the presence of (0.1 mM) chloroquine is shown. Each data point represents the mean and standard deviation of duplicate determinations.

FIGS. 9A and 9B. Comparison of internalization of isolated ^{125}I-fVIII and components of fVIII/vWf complex. Wells containing 2x10^{5} of each MEF and PEA 13 cells were incubated with 1 nM of isolated ^{125}I-fVIII or 1 nM of fVIII/vWf complex formed by mixing either ^{125}I-fVIII (1 nM) with unlabeled vWf (50 nM) or ^{125}I-vWf (50 nM) with unlabeled fVIII (1 nM). Following incubation for 6 hours with MEF cells in the absence of RAP (open bars) or in the presence of 1 μM RAP (solid bars) or after incubation with PEA 13 cells (hatched bars) the amounts of internalized (*panel A*) and degraded (*panel B*) isolated ^{125}I-fVIII, and ^{125}I-fVIII or ^{125}I-vWf from the fVIII/vWf complex were determined as described

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in Fig. 8. The data shown are an average of duplicate determinations ± standard deviation.

FIGS. 10A and 10B. The A2 domain of fVIII inhibits the internalization and degradation of ^{125}I-fVIII/vWf complex by MEF fibroblasts. One nM of ^{125}I-fVIII/vWf complex was prepared as in Fig. 8 and incubated with 2x10^5 of MEF cells in presence of 1 μM of A2 (○), 1 μM of Al/A3-Cl-C2 (Δ), or in the absence of any competitor (●). At the indicated times, the amounts of internalized (*panel A*) and degraded ^{125}I-fVIII (*panel B*) were determined as in Fig. 8. Each data point represents the mean and standard deviation of duplicate determinations.

FIGS. 11A-D. Internalization and degradation of ^{125}I-A2 by MEF fibroblasts and by LRP-expressing smooth muscle cells (SMC) and alveolar epithelial cells (T2). In the *panels A and B*, 2x10^5 of MEF (○, ●) or PEA 13 cells (Δ, ▲) were incubated with 10 nM ^{125}I-A2 in the absence (closed symbols) or presence (opened symbols) of RAP (1 μM). At the indicated times, the amounts of internalized ^{125}I-A2 (*panel A*) and degraded ^{125}I-A2 (*panel B*) by the MEF and PEA 13 fibroblasts were determined as described in Fig. 8. In the experiment (∇), degradation of ^{125}I-A2 by MEF cells in the presence (0.1 mM) chloroquine is shown. Each data point represents the mean and standard deviation of duplicate determinations. In the *panels C and D*, ^{125}I-A2 (10 nM) was incubated for 4 h at 37°C in the wells containing 3x10^5 SMC (solid bars) or T2 (open bars) cells in the presence or absence of RAP (1 mM). The amount of ^{125}I-A2 internalized (*panel C*) and degraded (*panel D*) by the cells was determined as in Fig. 8. The data shown are an average of duplicate determinations ± standard deviation.

FIGS. 12A and 12B. The effect of RAP on clearance of ^{125}I-A2 (A) or ^{125}I-fVIII/vWf (B) from plasma of mice. BALB/c mice were injected into the tail vein by sample containing ^{125}I-A2 (36 nM), *panel A*, or ^{125}I-fVIII/vWf (20 nM), *panel B*, in the absence (●) or presence (○) of RAP (267 μM). At indicated time points, blood (50 μl) was collected into 10 μl of 100 mM EDTA and an aliquot (50 μl) was counted for radioactivity. The percentage of ligand remaining in circulation was calculated considering radioactivity of the aliquot taken at 1 min

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after injection as 100%. The clearance of each preparation was examined in two mice, and the data plotted represent the average value ± standard deviation.

Detailed Description of the Preferred Embodiments

5

"Factor VIII" (or "coagulation factor VIII"), as used herein, refers to a plasma glycoprotein that is a member of the intrinsic coagulation pathway and is essential to blood coagulation. A congenital X-linked deficiency of biologically active factor VIII results in Hemophilia A, a potentially life-threatening disorder. Unless otherwise specified or indicated, as used herein, "factor VIII" denotes any functional human factor VIII protein molecule in its normal role in coagulation,

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including any fragment, analog derivative or modified factor VIII. The human factor VIII cDNA nucleotide and full-length predicted amino acid sequences are shown in SEQ ID NOs:1 and 2, respectively. Human factor VIII peptides of the invention include full-length factor VIII, full-length factor VIII minus Met at the N-terminus, mature factor VIII (minus the signal sequence), mature factor VIII

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with an additional Met at the N-terminus, and/or factor VIII with or without a B domain. Factor VIII of the invention may also include porcine factor VIII. The cDNA and predicted amino acid sequences of the porcine factor VIII are disclosed in U.S. Patent No.,859,204.

"Subunits" of factor VIII, as used herein, are the heavy and light chains of

20

the protein. The heavy chain of factor VIII contains three domains, A1, A2, and B. The light chain of factor VIII also contains three domains, A3, C1, and C2. Factor VIII is synthesized as an approximately 300 kDa single chain protein with internal sequence homology that defines the "domain" sequence NH_2 -A1-A2-B-A3-C1-C2-COOH.

25

In a factor VIII molecule, a "domain", as used herein, is a continuous sequence of amino acids that is defined by internal amino acid sequence identity and sites of proteolytic cleavage by thrombin. Unless otherwise specified, factor VIII domains include the following amino acid residues: A1, residues

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Ala1-Arg372; A2, residues Ser373-Arg740; B, residues Ser741-Arg1648; A3, residues Ser1690-Ile2032; C1, residues Arg2033-Asn2172; C2, residues Ser2173-Tyr2332. The A3-C1-C2 sequence includes residues Ser1690-Tyr2332. The remaining sequence, residues Glu1649-Arg1689, is usually referred to as the

5   factor VIII light chain activation peptide.

A "B-domainless" factor VIII or "B (-)" factor VIII, or fragment of thereof, as used herein, refers to any one of the factor VIII mutants described herein that lacks the B domain. The amino acid sequence of mature, B (-) factor VIII as constructed from GenBank Accession No. X01179 is shown in Figure 2 (SEQ ID

10   NO:5). B (-) factor VIII of the invention includes B (-) factor VIII with or without a signal sequence and with or without a Met at the N-terminus.

As used herein, a "mutant factor VIII or fragment thereof" or "factor VIII mutant or fragment thereof" is an active factor VIII molecule or fragment thereof comprising at least one amino acid substitution.

15   "RAP," as used herein, refers to the receptor-associated protein, also called the α_2 macroglobulin receptor-associated protein. RAP reduces receptor-dependent clearance of factor VIII. The human RAP deduced amino acid sequence is shown in Figure 4 (SEQ ID NO:4; GenBank Accession No. P30533). The RAP cDNA sequence is shown in SEQ ID NO:3 and GenBank Accession

20   No. M63959. Mutant RAP proteins of the invention may have an amino acid substitution at one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty or more positions of RAP. An amino acid substitution at "position" 327, for example, of RAP, refers to an amino acid substitution at amino acid 327 of the

25   RAP amino acid sequence in GenBank Accession No. P30533.

By "amino acid substitution" is meant a substitution of one amino acid for one of the remaining 19 naturally occurring amino acids. By an amino acid substitution at any one of positions "484 to 509," for example, is meant an amino acid substitution any position in the range, including at positions 484 and 509.

30   The mutant factor VIII or RAP proteins of the invention may have an amino acid

substitution at one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty or more positions.

An amino acid substitution at "position" 499, for example, of factor VIII, refers to an amino acid substitution at position 499 according to the numbering system of Wood *et al.*, *Nature 312*:330-337 (1984).

"Half-life," as used herein, refers to the half-life of factor VIII in circulation, as determined in animals such as mice, for example, using the method of Examples 1 and 2. Factor VIII has a half-life of 12-14 hours. As provided herein, methods to increase the half-life of factor VIII would lead to a factor VIII half-life of longer than 12-14 hours.

"Receptor-dependant clearance," as used herein, refers to the receptor-mediated removal of factor VIII from circulation. As described in the examples, receptor-dependant clearance is exhibited by MEF cells, and is inhibited by RAP. Receptor-dependent clearance includes, but is not limited, to LRP-mediated clearance of factor VIII clearance. Additional receptors may be involved in receptor-dependent clearance.

"Receptor-independent clearance," as used herein, refers to the removal of factor VIII from circulation by means different from receptor-dependant clearance. RAP does not inhibit receptor-independent clearance.

"Factor VIII deficiency," as used herein, includes deficiency in clotting activity caused by production of defective factor VIII, by inadequate or no production of factor VIII, or by partial or total inhibition of factor VIII by inhibitors. Hemophilia A is a type of factor VIII deficiency resulting from a defect in an X-linked gene and the absence or deficiency of the factor VIII protein it encodes. A deficiency in vWf can also cause phenotypic hemophilia A because vWf is an essential component of functional factor VIII. In these cases, the half-life of factor VIII is decreased to such an extent that it can no longer perform its particular functions in blood-clotting.

"Plasma," as used herein, refers to the fluid, non-cellular portion of the blood of humans or animals as found prior to coagulation. It is distinguished from serum, which is obtained after coagulation.

"Pharmaceutically acceptable carrier," as used herein, refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

"Patient," as used herein, refers to human or animal individuals receiving medical care and/or treatment.

"Congenital deficiency," as used herein, refers to the condition of an individual that lacks, as a result of heredity, a compound found in normal individuals. Congenital deficiencies are permanent absent transplantation or genetic intervention, which at this time are not guaranteed cures.

"Acquired deficiency," as used herein, refers to the condition of an individual that lacks, as a result of a non-congenital influence, a compound found in normal individuals. Acquired deficiencies are frequently the transient result of other conditions or their treatment, but are nonetheless debilitating and life threatening.

A "fusion protein," as used herein, is the product of a gene in which the coding sequence for one protein is extensively altered, for example, by fusing part of it to the coding sequence for a second protein from a different gene to produce a gene that encodes the fusion protein. As used herein, a fusion protein is a subset of the factor VIII protein or RAP protein described in this application.

A "corresponding" nucleic acid or amino acid or corresponding sequence of either, as used herein, is one present at a site in a factor VIII or mutant factor VIII molecule or fragment thereof that has the same structure and/or function as a site in the factor VIII molecule of another species, although the nucleic acid or amino acid number may not be identical.

"Procoagulant activity," as used herein, refers to factor VIII coagulation activity exhibited in a human factor VIII assay.

"Specific activity," as used herein, refers to the activity that will correct the coagulation defect of human factor VIII deficient plasma. Specific activity is measured in units of clotting activity per milligram total factor VIII protein in a standard assay in which the clotting time of human factor VIII deficient plasma is compared to that of normal human plasma. One unit of factor VIII activity is the activity present in one milliliter of normal human plasma. In the assay, the shorter the time for clot formation, the greater the activity of the factor VIII being assayed. Mutant factor VIII has coagulation activity in a human factor VIII assay. This activity may be less than, equal to, or greater than that of either plasma-derived or recombinant human factor VIII.

"Polypeptides," "molecules" and "proteins," as used herein, includes all polypeptides as described below. The basic structure of polypeptides is well known and has been described in innumerable textbooks and other publications in the art. In this context, the term is used herein to refer to any peptide or protein comprising two or more amino acids joined to each other in a linear chain by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types.

It will be appreciated that polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids, and that many amino acids, including the terminal amino acids, may be modified in a given polypeptide, either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques which are well known to the art. Even the common modifications that occur naturally in polypeptides are too numerous to list exhaustively here, but they are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. Among the known modifications which may be present in polypeptides of the present invention are, to name an illustrative few, acetylation, acylation,

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ADP-ribosylation, amidation, PEGylation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formulation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

Such modifications are well known to those of skill and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as, for instance *Proteins – Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W.H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as, for example, those provided by Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pp. 1-12 in *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter *et al.*, Analysis for protein modifications and nonprotein cofactors, *Meth. Enzymol. 182*: 626-646 (1990) and Rattan *et al.*, *Protein Synthesis: Post translational Modifications and Aging*, Ann. N.Y. Acad. Sci. 663: 48-62 (1992).

In general, as used herein, the term polypeptide encompasses all such modifications, particularly those that are present in polypeptides synthesized by expressing a polynucleotide in a host cell.

The invention also relates to fragments, "derivatives" and analogs of these polypeptides. The terms "fragment," "derivative" and "analog" when referring to the polypeptides of FIGS. 2, 3 or 4, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. A mutant, fragment

derivative or analog of factor VIII refers to a polypeptide that retains factor VIII procoagulant activity. A mutant, fragment derivative or analog of RAP refers to a polypeptide that retains the ability to reduce receptor-dependent clearance of factor VIII. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide. Fragments, derivatives and analogs are described in detail herein.

A fragment, derivative or analog of the polypeptide of the invention may be (i) one in which one or more of the amino acid residues includes a substituent group, or (ii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iii) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide. In certain preferred embodiments it is a recombinant polypeptide.

Further particularly preferred in this regard are mutants, analogs and fragments; and mutants and analogs of the fragments, having the defined activity and/or having the amino acid sequence of the polypeptides of FIGS. 2, 3 or 4.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

"Polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single-and double-stranded DNA, DNA that is a mixture of single-and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising

DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide.

As used herein, the term polynucleotide includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein.

It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, inter alia.

Polynucleotides of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced by chemical synthetic techniques or by a combination thereof. The DNA may be double-stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

Polynucleotides of the present invention may include, but are not limited to the coding sequence for the mature polypeptide, by itself; the coding sequence for the mature polypeptide and additional coding sequences, such as those encoding a leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the

aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing--including splicing and polyadenylation signals, for example--ribosome binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexahistidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci.*, *USA 86*: 821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag corresponds to an epitope derived of influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell 37*: 767 (1984), for instance.

An "effective amount" of an agent, as used herein, is an amount of such agent that is sufficient to bring about a desired result, especially upon administration of such agent to an animal or human.

The term "administration" is meant to include introduction of polypeptides or polynucleotides of the invention into an animal or human by any appropriate means known to the medical art, including, but not limited to, injection, oral, enteral, transdermal and parenteral (e.g., intravenous) administration.

The term "pharmaceutically acceptable salt" is intended to include salts of the mutant factor VIII or RAP of the invention. Such salts can be formed from pharmaceutically acceptable acids or bases, such as, for example, acids such as sulfuric, hydrochloric, nitric, phosphoric, etc., or bases such as alkali or alkaline earth metal hydroxides, ammonium hydroxides, alkyl ammonium hydroxides, etc.

The term "pharmaceutically acceptable composition" is intended to include solvents, carriers, diluents, and the like, which are utilized as additives or vehicles to preparations of the mutant factor VIII or RAP of the invention so as

to provide a carrier or adjuvant for the administration of such compounds to patients (human or animal) in need of the same. Such additives can perform certain functions, such as, for example, provide the proper ionic conditions for administration, stabilize the mutant factor VIII or RAP against inactivation or degradation, and/or increase the half-life of the mutant factor VIII or RAP. A pharmaceutically acceptable composition is medically compatible with the host to which it is being administered.

The term "treatment" or "treating" is intended to include the administration of the pharmaceutically acceptable compositions of the invention comprising effective amounts of mutant factor VIII or RAP (polypeptides or polynucleotides) of the invention to a patient for purposes which may include prophylaxis, amelioration, prevention or cure of a medical disorder.

A material is said to be "substantially free of natural contaminants" if it has been substantially purified from materials with which it is normally and naturally found before such purification and those contaminants normally and naturally found with the substance *in vivo* or *in vitro* are substantially absent from the final preparation of the material. When administered to a subject in need of treatment, the mutant factor VIII or RAP of the invention is substantially free of natural contaminants which associate with the mutant factor VIII or RAP either *in vivo* (in the host from which the mutant factor VIII or RAP was isolated), or *in vitro* (as a result of a chemical synthesis). By "substantially absent" is meant that such contaminants are either completely absent or are present at such low concentrations that their presence (1) does not interfere with the desired therapeutic effect of the active agent in the therapeutically acceptable composition when such composition is administered to a patient in need of same and (2) does not harm the patient as the result of the administration of such composition.

Since current information indicates that the B domain has no known effect on factor VIII function, in some embodiments the B domain is deleted ("B domain (-)" or "B domainless") in the mutant factor VIII molecule or fragments

thereof ("B(-) factor VIII" or "B domainless factor VIII") prepared by any of the methods described herein.

Generation of mutant(s) with a prolonged lifetime may be a promising approach to increase the efficacy and reduce the cost of fVIII infusion therapy. The invention provides methods of increasing the half-life of factor VIII by mutating factor VIII, and further provides methods of increasing the half-life of factor VIII using receptor-associated protein (RAP).

Factor VIII Mutants: A2 Domain

A recombinant mutant factor VIII having reduced receptor-dependent clearance and/or reduced receptor-independent clearance, and/or having superior coagulant activity, compared to human factor VIII, may be less expensive to make than plasma-derived factor VIII and may decrease the amount of factor VIII required for effective treatment of factor VIII deficiency.

The present invention provides active recombinant mutant factor VIII molecules or fragments thereof comprising at least one amino acid substitution in the A2 domain, polynucleotides encoding these, methods of producing and isolating them, and methods for characterizing their coagulant and plasma clearance properties.

The A2 domain is necessary for the procoagulant activity of the factor VIII molecule. Studies show that porcine factor VIII has six-fold greater procoagulant activity than human factor VIII (Lollar, P., and E. T. Parker 266 J. Biol. Chem. 12481-12486 (1991)), and that the difference in coagulant activity between human and porcine factor VIII appears to be based on a difference in amino acid sequence between one or more residues in the human and porcine A2 domains (Lollar, P., et al., 267 J. Biol. Chem. 23652-23657 (1992)).

In one embodiment, the invention provides a method of increasing the half-life of factor VIII by substituting amino acids in the factor VIII A2 domain. In another embodiment, the invention provides mutant factor VIII and fragments thereof, and the polynucleotides encoding same, which have an increased

circulating half-life than human factor VIII. The increased circulating half-life is due to a reduction in receptor-dependent clearance of factor VIII. As shown in the examples, amino acids in the factor VIII A2 domain interact with at least one receptor that mediates A2 clearance and factor VIII clearance from plasma.

Thus, factor VIII mutants of the invention include mutants with one or more substitutions within the A2 domain. In a preferred embodiment, the factor VIII mutants have an amino acid substitution at one or more positions from 484 to 509. This region includes the following sequence: NH_2- Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe -COOH.

In another preferred embodiment, the factor VIII mutants have an amino acid substitution at one or more of positions 484, 489, 490, 493, 496 or 499.

The amino acid at a particular position is substituted with any of the 19 other naturally occurring amino acids. A2 amino acid substitutions of the invention are those that inhibit the interaction of factor VIII with its clearance receptor(s). Thus, nonconservative A2 amino acid substitutions are preferred over conservative substitutions. Conservative amino acid substitutions include, for example, the substitution of an acidic amino acid with another acidic amino acid, a basic amino acid with another basic amino acid, a hydrophobic amino acid with a another hydrophobic amino acid, a polar amino acid with another polar amino acid, or an aromatic amino acid with another aromatic amino acid. Conservative amino acid substitutions are well known in the art.

Thus, an example of a conservative substitution is the substitution of Lys with Arg, while an example of a preferred nonconservative substitution is the substitution of Lys with Asp, Glu, Tyr, Asn, Gln, Thr, Ser, Cys, Trp, Phe, Pro, Met, Val, Leu, Ile, Trp, Gly or Ala.

Preferred A2 amino acid substitutions of the invention are the substitution of Lys or Arg with Leu, Ile or Val. Additional preferred A2 amino acid substitutions of the invention are the substitutions of Lys or Arg with Asp or Glu.

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Further preferred amino acid substitutions of the invention are the substitution of Lys or Arg with Ala, Ser, Thr, Met or Gly.

In another embodiment, amino acids at positions outside 484-509 are substituted, such as at positions 480, 481, 482, 483, 510, 511, 512 or 513. Preferred substitutions at these positions are those that reduce receptor-dependent clearance of factor VIII, such as introducing bulky or negatively charged amino acids.

Specifically provided as an exemplary and a preferred embodiment is active recombinant human factor VIII having substituted amino acids in the A2 domain, the polynucleotide encoding it, and the methods of producing, isolating, and characterizing its activity. The methods by which this mutant is prepared can also be used to prepare active recombinant factor VIII or fragments thereof having substituted amino acids in domains other than A2. One skilled in the art will recognize that these methods also demonstrate how other recombinant mutant factor VIII molecules or fragments thereof can be prepared in which amino acids are substituted. Additionally, recombinant methods are described in *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, eds. (1991); and Sambrook, J., *et al.*, Molecular Cloning. A Laboratory Manual.

Mutant factor VIII is prepared starting with human cDNA (Biogen, Inc.) encoding the factor VIII sequence. In a preferred embodiment, the factor VIII encoded by this cDNA includes domains A1-A2-A3-C1-C2, lacking the entire B domain, and corresponds to amino acid residues 1-740 and 1649-2332 of single chain human factor VIII (see SEQ ID NO:2), according to the numbering system of Wood *et al.*, 312 Nature 330-337 (1984).

The mutant factor VIII cDNA are cloned into expression vectors for ultimate expression of active factor VIII protein molecules in cultured cells by established techniques, as described by Selden, R.F., "Introduction of DNA into mammalian cells," in *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds (1991).

In a preferred embodiment, a cDNA encoding mutant factor VIII is inserted in a mammalian expression vector, such as ReNeo, to form a mutant factor VIII construct. Preliminary characterization of the mutant factor VIII is accomplished by insertion of the mutant cDNA into the mammalian expression

5 vector and transient expression of the mutant protein in COS-7 cells. A determination of whether active protein is expressed can then be made. The expression vector construct is used further to stably transfect cells in culture, such as baby hamster kidney cells, using methods that are routine in the art, such as liposome-mediated transfection (Lipofectin™, Life Technologies, Inc.).

10 Expression of recombinant mutant factor VIII protein can be confirmed, for example, by sequencing, Northern and Western blotting, or polymerase chain reaction (PCR). Mutant factor VIII protein in the culture media in which the transfected cells stably expressing the protein are maintained can be precipitated, pelleted, washed, and resuspended in an appropriate buffer, and the recombinant

15 mutant factor VIII protein purified by standard techniques, including immunaffinity chromatography using, for example, monoclonal anti-A2-Sepharose™.

In a further embodiment, the mutant factor VIII comprising amino acid substitutions is expressed as a fusion protein from a recombinant molecule in

20 which sequence encoding a protein or peptide that enhances, for example, stability, secretion, detection, isolation, or the like is inserted in place adjacent to the factor VIII encoding sequence. Established protocols for use of homologous or heterologous species expression control sequences including, for example, promoters, operators, and regulators, in the preparation of fusion proteins are

25 known and routinely used in the art. (See *Current Protocols in Molecular Biology*, Ausubel, F.M., *et al.*, eds, Wiley Interscience, N.Y.)

Other vectors, including both plasmid and eukaryotic viral vectors, may be used to express a recombinant gene construct in eukaryotic cells depending on the preference and judgment of the skilled practitioner (see, for example,

30 Sambrook *et al.*, Chapter 16). Other vectors and expression systems, including

bacterial, yeast, and insect cell systems, can be used but are not preferred due to differences in, or lack of, glycosylation.

The purified mutant factor VIII or fragment thereof can be assayed for amount and for coagulation activity by standard assays including, for example, the plasma-free factor VIII assay, the one-stage clotting assay, and the enzyme-linked immunosorbent assay using purified recombinant human factor VIII as a standard.

Recombinant mutant factor VIII protein can be expressed in a variety of cells commonly used for culture and recombinant mammalian protein expression. A preferred cell line, available from the American Type Culture Collection, Rockville, Md., is baby hamster kidney cells, which are cultured using routine procedure and media.

Any mutant factor VIII construct having an amino acid substitution at one or more positions in the A 2 domain as described can be assayed by standard procedures for coagulant activity and may be assayed for receptor-dependent clearance as described herein to identify mutant factor VIII molecules with enhanced coagulant activity and/or reduced receptor-mediated clearance. Mutant molecules may also be identified that have reduced coagulant activity compared to human or porcine factor VIII but also have reduced receptor-mediated clearance. One skilled in the art will recognize that mutant factor VIII molecules or fragments thereof having less, equal, or greater coagulant activity, compared to human or porcine factor VIII, is useful for treating patients who have a factor VIII deficiency. The methods described herein to prepare active recombinant mutant factor VIII with amino acid substitution(s) in the A2 domain can be used to prepare active recombinant mutant factor VIII protein with amino acid substitution(s) in the C2 domain or fragments thereof.

These molecules can be expressed in COS-7 cells and baby hamster kidney cells as described above. They can be purified to homogeneity using methods known in the art, such as heparin-Sepharose™ and immunoaffinity chromatography. Protein concentration can be estimated by absorption of

ultraviolet light at A_{280}, and the specific activity of the constructs can be determined by dividing coagulant activity (measured in units per ml by single stage clotting assay) by A_{280}. Human factor VIII has a specific activity of approximately 3000-4000 U/A_{280}, whereas porcine factor VIII has a specific activity of approximately 20,000 U/A_{280}. In a preferred embodiment, the coagulant mutant factor VIII has a specific activity of 3000 U/A_{280}. In a preferred embodiment, the coagulant mutant factor VIII has a specific activity of 3000 U/A_{280}. The a specific activity of mutant factor VIII may be anywhere in the range of 1000-20,000 U/A_{280}.

As described herein, site-directed mutagenesis techniques are used to identify mutant protein with coagulant activity that can be enhanced, equal to, or reduced, compared to human factor VIII, but preferably is enhanced. Oligonucleotide-directed mutagenesis can be used as described in Kunkel, T.A., et al., *Meth. Enzymol. 204*:125-139 (1991).

The mutant factor VIII proteins of the invention may have an amino acid substitution at one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, twenty or more positions of factor VIII. The mutant factor VIII molecules of the invention may have amino acid substitutions in more than one domain, such as having an amino acid substitution both in the A2 domain and in the C2 domain.

The present invention contemplates that mutant factor VIII cDNA and protein can be characterized by methods that are established and routine, such as DNA sequencing, coagulant activity assays, mass by ELISA and by UV absorbency at 280 nm of purified mutant factor VIII, specific coagulant activity (U/mg), SDS-PAGE of purified mutant factor VIII, and the like. Other known methods of testing for clinical effectiveness may be required, such as amino acid, carbohydrate, sulfate, or metal ion analysis.

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Factor VIII Mutants: C2 Domain

The same methods employed for preparing mutant human factor VIII having A2 domain amino acid substitution(s) can be used to prepare other recombinant mutant factor VIII protein and fragments thereof and the polynucleotides encoding these, such as mutant factor VIII having amino acid substitutions in the C2 domain.

Mutant human factor VIII molecules with amino acid substitution(s) in the C2 domain, which have reduced or no receptor-independent clearance can be identified. More specifically, the procedures can be the same or similar to those described herein for amino acid substitution in the A2 domain (by alanine scanning mutagenesis, site-directed mutagenesis, etc.,) substituting amino acids in the C2 domain of B (-) factor VIII; insertion into an expression vector, such as pBluescript; expression in cultured cells; and routine assay for coagulant activity and receptor-independent clearance.

In one embodiment, the invention provides mutant factor VIII and fragments thereof, and the polynucleotides encoding same, which have an increased circulating half-life than human factor VIII. The increased circulating half-life of mutant factor VIII is due to a reduction in receptor-independent clearance of factor VIII.

The C2 domain consists of amino acid residues 2173-2332. Within this 154 amino acid region, positions 2303-2332 are involved in both phospholipid binding and vWf binding. A synthetic peptide of factor VIII amino acids 2310-2320 (in which residues 2310 and 2320 are covalently linked) competes with factor VIII for phospholipid binding. A comparison of factor V, which does not bind vWf, and factor VIII reveals 5 amino acids within positions 2311-2319 that are unique to factor VIII. Although not being bound by any theory, these unique positions (Gln2311, Ser 2312, Val 2314, His2315 and Gln2316) are important for receptor-independent clearance, but are not critical for vWf binding.

Thus, one embodiment of the present invention is a mutant factor VIII having an amino acid substitution at one or more of positions 2173-2332 in the

C2 domain. In another preferred embodiment, the mutant factor VIII has an amino acid substitution at one or more positions 2311-2319 in the C2 domain.

The amino acid at a particular position is substituted with any of the 19 other naturally occurring amino acids. C2 amino acid substitutions of the invention are those that inhibit the interaction of factor VIII with phospholipid. Thus, nonconservative C2 amino acid substitutions are preferred over conservative substitutions. Conservative amino acid substitutions include, for example, the substitution of an acidic amino acid with another acidic amino acid, a basic amino acid with another basic amino acid, a hydrophobic amino acid with a another hydrophobic amino acid, a polar amino acid with another polar amino acid, or an aromatic amino acid with another aromatic amino acid. Conservative amino acid substitutions are well known in the art.

Thus, an example of a conservative substitution is the substitution of Leu with Ile or Val, while an example of a preferred nonconservative substitution is the substitution of Leu with Asp, Glu, Arg, Lys, His, Tyr, Asn, Gln, Thr, Ser, Cys, Trp, Phe, Pro, Met, Trp, Gly or Ala. One preferred substitution is Ala.

Additional embodiments of the present invention include a method of treating hemophilia by administering a C2 domain mutant of factor VIII, pharmaceutically acceptable compositions comprising a C2 domain mutant of factor VIII either alone or in combination with RAP, and polynucleotides encoding a C2 domain mutant of factor VIII.

Furthermore, the amino acid substitution(s) in the C2 domain can be combined with amino acid substitution(s) in the A2 domain, to produce a mutant factor VIII with increased half-life.

Receptor Associated Protein

A preferred embodiment of the present invention is directed to a method of increasing the half-life of factor VIII by administering RAP. Preferably, the RAP binds LRP, more preferably, the RAP has an increased affinity for LRP as compared to the naturally occurring RAP.

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In another preferred embodiment of the present invention, RAP is a fragment, mutant or analog. Preferably, the RAP fragment, mutant or analog retains LRP binding activity. More preferably, the RAP fragment, mutant or analog has increased affinity for LRP as compared to the naturally occurring RAP.

In one embodiment, the RAP is a fragment having LRP binding activity. Such RAP fragments may comprise 10, 20, 30, 40, 50, 60, 75, 100, 125, 150, 175, 200, 250, 300 or 350 or more contiguous amino acids.

In one embodiment, RAP comprises amino acids 1 to 357 of Figure 4 (full-length RAP; amino acids -19 to 323 of SEQ ID NO:4). RAP contains a signal sequence 34 amino acids in length. Thus, in another embodiment, RAP comprises amino acids 35 to 357 of Figure 4 (mature RAP; amino acids 1 to 323 of SEQ ID NO:4.).

In another embodiment of the present invention, RAP contains an N-terminal or a C-terminal deletion, or a combination of N- and C-terminal deletions. N-terminal deletions often result in a protein with increased stability. Thus, for example, deleting between 1 and 50 amino acids from the N-terminus of mature RAP is useful to produce a more stable RAP. Therefore, additional embodiments of the present invention include, for example, RAP comprising amino acids 36-357, 37-357, 38-357, 39-357, 40-357, 41-357, 42-357, 43-357, 44-357, 45-357, 46-357, 47-357, 48-357, 49-357, 50-357, 51-357, 52-357, 53-357, 54-357, 55-357, 56-357, 57-357, 58-357, 59-357, 60-357, 61-357, 62-357, 63-357, 64-357, 65-357, 66-357, 67-357, 68-357, 69-357, 70-357, 71-357, 72-357, 73-357, 74-357, 75-357, 76-357, 77-357, 78-357, 79-357, 80-357, 81-357, 82-357, 83-357, 84-357 and 85-357 of Figure 4 (positions 1-323, 2-323, 3-323, 4-323, 5-323, 6-323, 7-323, 8-323, 9-323, 10-323, 11-323, 12-323, 13-323, 14-323, 15-323, 16-323, 17-323, 18-323, 19-323, 20-323, 21-323, 22-323, 23-323, 24-323, 25-323, 26-323, 27-323, 28-323, 29-323, 30-323, 31-323, 32-323, 33-323, 34-323, 35-323, 36-323, 37-323, 38-323, 39-323, 40-323, 41-323, 42-323.

43-323, 44-323, 45-323, 46-323, 47-323, 48-323, 49-323 and 50-323 of SEQ ID NO:4).

The LDL receptor binding domain encompasses amino acids 237 to 353 of Figure 4 (amino acids 203 to 319 of SEQ ID NO:4). Thus, a preferred embodiment of the present invention is RAP comprising amino acids 237 to 353 (amino acids 203 to 319 of SEQ ID NO:4).

Another embodiment of the present invention is a polynucleotide encoding RAP.

In another embodiment of the present invention, RAP or a polynucleotide encoding RAP is used to treat hemophilia either alone or in combination with a factor VIII mutant.

Additional embodiments of the present invention include pharmaceutically acceptable compositions comprising RAP alone or in combination with one or more factor VIII mutants.

Pharmaceutically Acceptable Compositions

Pharmaceutically acceptable compositions comprising mutant factor VIII or RAP, alone or in combination with appropriate pharmaceutical stabilization compounds, delivery vehicles, and/or carrier vehicles, are prepared according to known methods, as described in *Remington's Pharmaceutical Sciences* by E.W. Martin.

In one preferred embodiment, the preferred carriers or delivery vehicles for intravenous infusion are physiological saline or phosphate buffered saline.

In another preferred embodiment, suitable stabilization compounds, delivery vehicles, and carrier vehicles include but are not limited to other human or animal proteins such as albumin.

Phospholipid vesicles or liposomal suspensions are also preferred as pharmaceutically acceptable carriers or delivery vehicles. These can be prepared according to methods known to those skilled in the art and can contain, for example, phosphatidylserine/phosphatidylcholine or other compositions of

phospholipids or detergents that together impart a negative charge to the surface, since factor VIII binds to negatively charged phospholipid membranes. Liposomes may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the mutant factor VIII or RAP is then introduced into the container. The container in then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

Mutant factor VIII or RAP can be combined with other suitable stabilization compounds, delivery vehicles, and/or carrier vehicles, including vitamin K dependent clotting factors. tissue factor, and von Willebrand factor (vWf) or a fragment of vWf that contains the factor VIII binding site, and polysaccharides such as sucrose.

Mutant factor VIII can be stored bound to vWf to increase the shelf-life of the mutant molecule. Additionally, lyophilization of factor VIII can improve the yield of active molecules in the presence of vWf. Lyophilization can also improve the yield of RAP. Current methods for storage of human and animal factor VIII used by commercial suppliers can be employed for storage of mutant factor VIII or RAP. These methods include: (1) lyophilization of factor VIII in a partially-purified state (as a factor VIII "concentrate" that is infused without further purification); (2) immunoaffinity-purification of factor VIII by the Zimmerman method and lyophilization in the presence of albumin, which stabilizes the factor VIII; (3) lyophilization of recombinant factor VIII in the presence of albumin.

Additionally, factor VIII has been indefinitely stable at 4°C in 0.6 M NaCl, 20 mM MES, and 5 mM CaCl$_2$ at pH 6.0 and also can be stored frozen in these buffers and thawed with minimal loss of activity.

Methods of Treatment

Mutant factor VIII or RAP is used to treat uncontrolled bleeding due to factor VIII deficiency (e.g., intraarticular, intracranial, or gastrointestinal hemorrhage) in hemophiliacs with and without inhibitory antibodies and in patients with acquired factor VIII deficiency due to the development of inhibitory antibodies. The active materials are preferably administered intravenously.

Factor VIII is classically defined as that substance present in normal blood plasma that corrects the clotting defect in plasma derived from individuals with hemophilia A. The coagulant activity *in vitro* of purified and partially-purified forms of factor VIII is used to calculate the dose of factor VIII for infusions in human patients and is a reliable indicator of activity recovered from patient plasma and of correction of the *in vivo* bleeding defect. There are no reported discrepancies between standard assay of novel factor VIII molecules *in vitro* and their behavior in the dog infusion model or in human patients, according to Lusher, J. M., *et al., New. Engl. J. Med. 328*:453-459 (1993); Pittman, D. D., *et al., Blood 79*:389-397 (1992), and Brinkhous *et al., Proc. Natl. Acad. Sci. 82*:8752-8755 (1985).

Usually, the desired plasma factor VIII level to be achieved in the patient through administration of the mutant factor VIII is in the range of 30-100% of normal. In a preferred mode of administration of the mutant factor VIII, the composition is given intravenously at a preferred dosage in the range from about 5 to 50 units/kg body weight, more preferably in a range of 10-50 units/kg body weight, and most preferably at a dosage of 20-40 units/kg body weight; the interval frequency is in the range from about 8 to 24 hours (in severely affected hemophiliacs); and the duration of treatment in days is in the range from 1 to 10 days or until the bleeding episode is resolved. See, e.g., Roberts, H. R., and M. R. Jones, "Hemophilia and Related Conditions – Congenital Deficiencies of Prothrombin (Factor II, Factor V, and Factors VII to XII)," Ch. 153, 1453-1474, 1460, in *Hematology*, Williams, W. J., *et al.*, ed. (1990).

Administration of an effective amount of RAP will result in similar levels of factor VIII in patient blood as indicated above. Patients with inhibitors may require more mutant factor VIII, or patients may require less mutant factor VIII because of its higher specific activity than human factor VIII or increased plasma half-life. Likewise, patients may require more or less RAP, depending on RAP's binding affinity to LRP or other factor VIII clearance receptor, or depending on its stability in circulating blood. As in treatment with human or porcine factor VIII, the amount of mutant factor VIII or RAP infused is defined by the one-stage factor VIII coagulation assay and, in selected instances, *in vivo* recovery is determined by measuring the factor VIII in the patient's plasma after infusion. It is to be understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

Administration

In a preferred embodiment, pharmaceutically acceptable compositions of mutant factor VIII or RAP alone or in combination with stabilizers, delivery vehicles, and/or carriers are infused into patients intravenously according to the same procedure that is used for infusion of human or animal factor VIII.

The treatment dosages of mutant factor VIII or RAP composition that must be administered to a patient in need of such treatment will vary depending on the severity of the factor VIII deficiency. Generally, dosage level is adjusted in frequency, duration, and units in keeping with the severity and duration of each patient's bleeding episode. Accordingly, the mutant factor VIII or RAP is included in the pharmaceutically acceptable carrier, delivery vehicle, or stabilizer in an amount sufficient to deliver to a patient a therapeutically effective amount of the mutant protein to stop bleeding, as measured by standard clotting assays.

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Treatment can take the form of a single intravenous administration of the composition or periodic or continuous administration over an extended period of time, as required. Alternatively, mutant factor VIII or RAP can be administered subcutaneously or orally with liposomes in one or several doses at varying intervals of time. Mutant factor VIII or RAP can also be used to treat uncontrolled bleeding due to factor VIII deficiency in hemophiliacs who have developed antibodies to human factor VIII.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogens, e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, *J. Biomed. Mater. Res.* *15*:167-277 (1981) and Langer, *Chem. Tech.* *12*: 98-105 (1982) or poly(vinylalcohol), polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, *Biopolymers 22*:547-556 (19831)), non-degradable ethylene-vinyl acetate (Langer *et al.*, supra), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988). While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37 °C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization can be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release blood factor compositions also include liposomally entrapped blood factor or antibody. Liposomes containing the claimed blood factor or antibody are prepared by methods known per se: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. USA, 82: 3688-3692 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. USA, 77: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Pat. No. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamelar type, the selected proportion being adjusted for the optimal blood factor therapy. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556. Additionally, Giles, A. R., *et al. Brit. J. Hematol. 69*:491-497 (1988) describe the formulation of factor Xa in phosphatidylcholine-phosphatidylserine vesicles.

Additionally, mutant factor VIII or RAP can be administered by transplant of cells genetically engineered to produce the protein or by implantation of a device containing such cells, as described below.

Gene Therapy

Polynucleotides encoding the mutant factor VIII or RAP may be employed in accordance with the present invention by expression of such mutant factor VIII or RAP *in vivo*, in treatment modalities often referred to as "gene therapy."

Mutant factor VIII or RAP can also be delivered by gene therapy in the same way that human factor VIII can be delivered, using delivery means such as retroviral vectors. This method consists of incorporation of factor VIII cDNA into human cells that are transplanted directly into a factor VIII deficient patient or that are placed in an implantable device, permeable to the factor VIII molecules but impermeable to cells, that is then transplanted. The preferred method will be retroviral-mediated gene transfer. In this method, an exogenous gene (e.g., a factor VIII cDNA) is cloned into the genome of a modified retrovirus. The gene/cDNA is inserted into the genome of the host cell by viral machinery where it will be expressed by the cell. The retroviral vector is modified so that it will

not produce virus, preventing viral infection of the host. The general principles for this type of therapy are known to those skilled in the art and have been reviewed in the literature (e.g., Kohn, D.B., and P.W. Kantoff, *Transfusion* *29*:812-820 (1989)).

Thus, for example, cells from a patient may be engineered with a polynucleotide, such as a DNA or RNA, encoding a polypeptide ex vivo, and the engineered cells then can be provided to a patient to be treated with the polypeptide. For example, cells may be engineered ex vivo by the use of a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention. Such methods are well-known in the art and their use in the present invention will be apparent from the teachings herein.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by procedures known in the art. For example, a polynucleotide of the invention may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct then may be isolated and introduced into a packaging cell is transduced with a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention.

Retroviruses from which the retroviral plasmid vectors herein above mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

Such vectors well include one or more promoters for expressing the polypeptide. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller *et al., Biotechniques 7*: 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, RNA polymerase III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, Y-2, Y-AM, PA12, T19-14X, VT-19-17-H2, YCRE, YCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, A., *Human Gene Therapy 1*:5-14 (1990). The vector may be transduced into the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO$_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line will generate infectious retroviral vector particles, which include the polynucleotide(s) encoding the polypeptides. Such retroviral vector particles then may be employed to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the polynucleotide(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

The following examples are illustrative only and are not intended to limit the scope of the invention as defined by the appended claims. It will be apparent

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to those skilled in the art that various modifications and variations can be made in the methods of the present invention without departing from the spirit and scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the 5 scope of the appended claims and their equivalents.

All patents, publications and publicly available sequences referred to herein are expressly incorporated by reference.

Examples

Example 1

10 Activated factor VIII (fVIIIa) functions in the intrinsic pathway of blood coagulation as a cofactor for factor IXa in the conversion of factor X to activated factor X (Xa). When IXa is bound to membrane and fVIII the rate of factor X to IXa conversion increases 100,000-1,000,000 fold. The procoagulant activity of fVIIIa is regulated by rapid and potentially reversible dissociation of the A2 15 subunit from the A1/A3C1C2 dimer and by activated protein C (APC) proteolysis of the residual fVIIIa. Removal of the A2 and A1/A3C1C2 fragments is an additional *in vivo* mechanism to control factor VIIIa activity at the site of blood coagulation.

We tested this in a model system using mouse embryonic fibroblasts 20 (MEF) that express low density lipoprotein receptor related protein (LRP) a multi ligand endocytic receptor and PEA 13 fibroblasts that are genetically deficient in LRP. Using the above model system we studied the mechanisms of cellular uptake and degradation of thrombin activated fVIII subunits to evaluate the role of these mechanisms in regulation of fVIIIa level.

Methods

Cell mediated ligand internalization and degradation assays. Cells were seeded into 24 well dishes and allowed to grow for 24 hours at 37°C. 5% CO_2 MEF and PEA 13 cells were incubated for selected time intervals at 37°C with ^{125}I-labeled fVIIIa fragments in the presence and absence of unlabeled competitors as described in the figure legends. Radioactivity appearing in the cell culture medium that was soluble after precipitation with 10% trichloroacetic acid (TCA) was taken to represent degraded ligand. Total ligand degradation was corrected by subtracting the amount of 10% TCA soluble radioactivity occurred in control wells lacking cells. The amount of labeled ligand bound to the cell surface or that was internalized by cells was determined as follows. Cells were washed with cold phosphate buffered saline and treated with a trypsin EDTA proteinase K solution. Surface bound material was defined as the amount of radioactive ligand released by this treatment and the amount of internalized ligand was defined as the amount of radioactivity which remained associated with the cell pellet following the treatment.

Determining of the A2 affinity for LRP. LRP (3.5 μg/ml) in 0.1 M $NaHCO_3$, pH 9.6 was incubated in Immulon I microtiter well strips for 16 hours at 4°C. After washing with TBS, 5 mM $CaCl_2$, 0.05% Tween 20 buffer (TBS-T) and blocking with 3% BSA, ^{125}I-A2 (5 nM) and increasing concentrations unlabeled A2 (0-1750 nM) were added. Following the incubation for 1 hour at 37°C and washing with TBS-T, the radioactivity bound to the wells was counted. ^{125}I-A2 binding in the presence of unlabeled A2 was plotted using the computer program "Ligand." The K_d value for A2/LRP binding was calculated from the displacement curve, showing a best fit of the data to a single class of sites.

Effect of RAP on the clearance of ^{125}I-A2 domain from the plasma of mice. To elucidate the role of LRP receptor in the clearance of the A2 domain from

plasma *in vivo* we tested the plasma level of ^{125}I-labeled A2 in the presence and absence of RAP after tail vein injection in mice. 250 µl samples of A2 (36 nM), in the presence and absence of RAP (267 µM) were injected into the tail vein of BALB/c mice. At the indicated times, blood (50 µl) was collected into 10 µl of

5   0.5 M EDTA and counted for its ^{125}I content. RAP significantly delays the plasma elimination of A2 domain. This experiment indicates that a RAP dependent hepatic receptor, LRP, plays a major role in the removal of A2 from circulation.

LRP receptor mediated internalization and degradation of the ^{125}I-A2

10   **domain by fibroblast cells.** The cellular uptake and degradation of activated factor VIII fragments was studied using mouse embryonic fibroblast (MEF) cells expressing low density lipoprotein receptor - related protein (LRP), a multi ligand endocytic receptor, and PEA 13 cells represents fibroblasts lacking LRP. The fVIIIa subunits interaction with MEF and PEA 13 cells represent an adequate

15   model for *in vivo* processes because fibroblast cells became exposed to coagulation site upon vascular injury. LRP mediated internalization and degradation of some proteins (Thrombin:ATIII complex and other complexes of thrombin with inhibitors, tissue factor pathway inhibitor involved in coagulation cascade is known.

20   ^{125}I-A2 (10 nM) was incubated with cells for several times and amount of surface bound, internalized and degraded ^{125}I-labeled protein were determined as described under "Methods." The A2 domain was internalized and degraded by MEF cells but not by PEA 13 cells suggesting that expression of LRP receptor is required for these processes. The internalization and degradation of A2 was

25   blocked by RAP, an inhibitor of LRP binding to its ligands.

Internalization of the ^{125}I-A2 and APC cleaved A2 domain, by LRP presenting MEF cells and control PEA 13 cells, lacking LRP. Inactivation of fVIIIa by APC leads to a cleavage of the A2 at Arg^{562}. Since cofactor activity

cannot be reconstituted from $A2_N/A2_C$ and Al/A3C1C2 dimer, we proposed that $A2_N/A2_C$ removal from circulation may occur by a mechanism different than that for intact A2. To examine the effect of proteolysis by APC on cellular internalization of the A2 domain, we compared the ^{125}I-A2 and ^{125}I-$A2_N/A2_C$ uptake by MEF and PEA 13 cells. We found that in contrast to A2 domain, the internalization of ^{125}I-$A2_N/A2_C$ is not mediated by LRP receptor.

Binding the A2 domain to the immobilized LRP. To the microtiter wells with immobilized LRP ^{125}I-A2 (5 nM) and increasing concentrations of unlabeled A2 (0-1750 nM) were added. After incubation for 1 hour at 37°C the wells were washed with TBS-T and radioactivity bound to the wells was counted. ^{125}I-A2 binding in the presence of unlabeled A2 is expressed as the percentage of ^{125}I-A2 binding, when no competitor was added. The data was analyzed using the computer program "Ligand". The K_d value for A2/ LRP binding calculated from the displacement data was 130 nM.

Internalization of ^{125}I-labeled Al/A3ClC2 and Al^{336}/A3ClC2 by fibroblast cells. We proposed that phospholipid binding site previously localized to the C2 domain of fVIII light chain mediates the cellular surface binding and internalization of Al/A3ClC2 and Al^{336}/A3ClC2 dimers. To test this hypothesis we determined internalization ^{125}I-Al/A3ClC2 and ^{125}I-Al^{336}/A3ClC2 by MEF cells in the presence and absence of anti-C2 domain monoclonal antibody NMC-VIII/5, which blocks the membrane binding sites of the C2 domain.

Wells containing 2×10^5 MEF cells were incubated with 3 nM of ^{125}I-Al/A3ClC2 or 3 nM of ^{125}I-Al^{336}/A3ClC2 at 37°C in the presence or absence of 30 nM monoclonal antibody NMC-VIII/5. In the control experiments, PEA 13 cells lacking LRP were incubated as above with ^{125}I-Al/A3ClC2 and ^{125}I-Al^{336}/A3ClC2. At several times internalization of the dimers was described under "Methods."

Since internalization of both ^{125}I-Al/A3C1C2 and ^{125}I-Al^{336}/A3ClC2 dimers was completely inhibited hy monoclonal antibody NMC-VIII/5, that recognizes the membrane binding site of fVIII C2 domain, we concluded that membrane binding of C2 is a critical step required for internalization of the above dimers. The rate of internalization was similar for MEF and PEA 13 cells, which indicates that LRP receptor is not involved in this process.

Degradation of ^{125}I-Al/A3C1C2 and ^{125}I-Al^{336}/A3ClC2 by MEF cells. MEF cells were incubated with ^{125}I-Al/A3ClC2 (3 nM) or ^{125}IAl^{336}/A3ClC2 (3 nM) for 22 hours at 37°C in the presence and absence PAP (1 μM). The degradation of dimers was measured as described under "Methods".

The degradation of Al/A3ClC2 dimer is RAP dependent. In contrast, degradation of APC cleaved Al^{336}/A3ClC2 dimer is RAP independent and does not correlate with LRP expression.

Conclusions

The A2 domain was internalized and degraded by mouse embryonic fibroblasts (MEF) which are expressing low density lipoprotein receptor - related protein (LRP), a multi ligand endocytic receptor. The internalization and degradation of A2 was blocked by RAP, an inhibitor of LRP binding to its ligands. *In vivo* clearance studies in mice demonstrated that RAP inhibited the clearance of ^{125}I-A2 from circulation. The radioactivity was preferentially accumulated in liver in the absence but not in the presence of RAP. This indicate that a RAP sensitive hepatic receptor most likely LRP, plays a major role in the removal of ^{125}I-A2 from the circulation.

The phospholipid binding site previously localized to the C2 domain of fVIII light chain mediates the cellular membrane binding and internalization of Al/A3ClC2 and Al^{336}/A3ClC2 dimers.

LRP receptor does not participate in cellular uptake and degradation of fragments $A2_N/A2_C$ and $A1^{336}/A3C1C2$, produced by irreversible inactivation of fVIIIa by APC. A2 and A1/A3C1C2 fragments produced by reversible inactivation of fVIIIa are removed by LRP-mediated and LRP-independent mechanisms, respectively. LRP is involved in the regulation of coagulation processes *in vivo*, by removal of A2 domain and A1/A3C1C2 dimer, the fragments from which active factor VIIIa can be reconstituted.

Example 2

The plasma glycoprotein factor VIII (fVIII) serves as a cofactor for the factor X activation complex in the intrinsic pathway of blood coagulation. FVIII circulates in plasma in a tight noncovalent complex with its carrier protein von Willebrand factor (vWf). Although the complex formation of fVIII with vWf is critical for maintenance of a normal half-life and level of fVIII in circulation, the mechanisms associated with fVIII turnover are not well defined. In the present study, we found that catabolism of fVIII is mediated by the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP), a liver endocytic, receptor responsible for *in vivo* clearance of a number of structurally unrelated ligands. A specific binding between fVIII and LRP was demonstrated by homologous ligand competition experiments, where a K_d of 116 nM was determined for fVIII binding to LRP. A 39 kDa receptor-associated protein (RAP), an antagonist of ligand binding by LRP, completely inhibited fVIII binding to purified LRP. The region of fVIII involved in its binding to LRP was localized to the A2 domain residues 484-509, based on the ability of the isolated A2 domain and the synthetic A2 domain peptide 484-509 to prevent fVIII interaction with LRP. Since vWf did not inhibit fVIII binding to LRP, we proposed that LRP receptor may internalize fVIII from its complex with vWf. In agreement with this, mouse embryonic fibroblasts (MEF) that express LRP, but not fibroblasts genetically deficient in LRP (PEA 13), were able to internalize and

degrade ^{125}I-fVIII/vWf complex. The latter processes were competed by RAP and A2 subunit of fVIII, indicating that cellular internalization and subsequent degradation were mediated by interaction of the A2 domain of fVIII with LRP. MEF cells were not able to internalize ^{125}I-vWf from ^{125}I-vWf /fVIII complex. This indicates that vWf does not follow fVIII in the LRP-mediated pathway and dissociates from fVIII at the early stage of endocytosis. *In vivo* clearance studies of ^{125}I-fVIII/vWf complex in mice demonstrated that RAP prolonged the half-life of ^{125}I-fVIII in circulation by 2.5-fold, indicating that RAP-sensitive receptor, most likely LRP, is responsible for the plasma clearance of fVIII.

Introduction

The plasma glycoprotein factor VIII (fVIII) functions as a cofactor for the factor X activation enzyme complex in the intrinsic pathway of blood coagulation, and it is decreased or nonfunctional in patients with hemophilia A. The fVIII protein consists of a homologous A and C domains and a unique B domain which are arranged in the order Al-A2-B-A3-Cl-C2 (Vehar, G.A., *et al.*, *Nature 312*:337-340 (1984)). It is processed to a series of Me^{2+} linked heterodimers produced by cleavage at the B-A3 junction (Fay, P. J., *et al.*, *Biochem. Biophys. Acta. 871*:268-278 (1986)), generating a light chain (LCh) consisting of an acidic region (AR) and A3, Cl, and C2 domains and a heavy chain (HCh) which consists of the Al, A2, and B domains (Fig. 1).

Transplantational studies both in animals and in humans demonstrated that the liver hepatocytes are the major fVIII-producing cells (Lewis, J. H., *et al.*, *N. Engl. J. Med 312*:1189-1191 (1985); Bontempo, F. A., *et al.*, *Blood 69*:1721-1724 (1987)). Immediately after release into circulation, fVIII binds with high affinity (K_d < 0.5 nM (MacGregor, I.R., *et al.*, *Vox. Sang. 69*:319-327 (1995); Saenko, E.L. and Scandella, D., *J. Biol Chem 272*:18007-18014 (1995)) to its carrier protein vWf to form a tight, noncovalent complex, which is required for maintenance of a normal fVIII level in the circulation. Complex formation with

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vWf stabilizes association of the LCh and HCh within fVIII molecule (Wise, R.J.,
et al., J. Biol. Chem. 266:21948-21955 (1991)) and prevents fVIII from C2-
domain mediated binding to phospholipid membranes (Gilbert, G.E., et al., J.
Biol. Chem. 267:1586115868 (1992)), activation by activated factor X

5   (Koppelman, S.J., et al., J. Lab. Clin. Med. 123:585-593 (1994)) and from
protein C-catalyzed inactivation (Fay, P.J., et al., J. Biol. Chem 266:2172-2177
(1991)). vWf comprises a series of high molecular weight, disulfide-bonded
multimers with molecular weight values as high as 2×10^7 Da (Hoyer, L.W. and
Shainoff, J.R., Blood 55:1056-1059 (1980)) and circulates in plasma at 10 μg/ml

10   or 50 nM, assuming a molecular mass of 270 kDa for vWf monomers (Girma, J.-
P., et al., Biochemistry 25:3156-3163 (1986)). Since the concentration of fVIII
in plasma is approximately 1 nM (Wion, K., et al., Nature 317:726-730 (1985)),
one fVIII molecule is bound per 50 vWf monomers (Vlot, A.J., et al., Blood
85:3150-3157 (1995)).

15   Activation of fVIII by thrombin leads to dissociation of activated fVIII
(fVIIIa) from vWf and to at least 100-fold increase of the cofactor activity. The
fVIIIa is a A1/A2/A3-C1-C2 heterotrimer (Fay, P.J., et al., J. Biol. Chem
266:8957-8962 (1991)) in which domains A1 and A3 retain the metal ion linkage
(Fig. 1) and the stable dimer A1/A3-C1-C2 is weakly associated with the A2

20   subunit through electrostatic forces (Fay, P.J., et al., J. Biol. Chem 266:8957-8962
(1991)). Spontaneous dissociation of the A2 subunit from the heterotrimer results
in non-proteolytic inactivation of fVIIIa.

                 Infusion of fVIII/vWf complex or purified plasma or recombinant fVIII
into patients with severe hemophilia A who do not have fVIII (Fijnvandraat, K.,

25   et al., Thromb. Haemostas. 77:298-302 (1997); Morfini, M., et al., Thromb.
Haemostas. 68:433-435 (1992)) or in normal individuals (Over, J., et al., J. Clin.
Invest. 62:223-234 (1978)) results in a similar fVIII disappearance with a half-life
of 12-14 hours. Although the complex between fVIII and vWf is crucial for
normal half-life and level of fVIII in the circulation, the mechanisms associated

30   with turnover of fVIII/vWf complex are not well defined. We proposed that

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fVIII/vWf complex is eliminated from plasma via clearance receptor and tested the possibility that this receptor is low density lipoprotein related protein receptor (LRP). Cellular endocytosis mediated by LRP was shown to be a mechanism of removal of a number of structurally unrelated ligands including several proteins

5   related to coagulation or fibrilolysis. These ligands are: complexes of thrombin with antithrombin III (ATIII), heparin cofactor II (HC11) (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 271*:6523-6529 (1996)), protease nexin I (Knauer, M.F., *et al.*, *J. Biol. Chem. 272*:12261-12264 (1997)), complexes of urokinase-type and tissue-type plasminogen activators (u-PA and t-PA, respectively) with plasminogen

10   activator inhibitor (PAI-1) (Nykjaer, A., *et al.*, *J. Biol. Chem. 267*:14543-14546 (1992);Orth, K., *et al.*, *Proc. Natl. Acad. Sci. 89*:7422-7426 (1992)), thrombospondin (Mikhailenko, I., *et al.*, *J. Biol. Chem. 272*:6784-6791 (1997)), tissue factor pathway inhibitor (TFPI) (Warshawsky, I., *et al.*, *Proc. Natl. Acad. Sci. 91*:6664-6668 (1994)), and factor Xa (Narita, M., *et al.*, *Blood 91*:555-560

15   (1998); Ho, G., *et al.*, *J. Biol. Chem 271*:9497-9502 (1996)).

LRP, a large cell-surface glycoprotein identical to α_2-macroglobulin receptor (Strickland, D.K., *et al.*, *J. Biol. Chem.* 265:17401-17404 (1990)), is a member of the low density lipoprotein (LDL) receptor family which also includes the LDL receptor, very low density lipoprotein (VLDL) receptor, vitellogenin

20   receptor and glycoprotein 330 receptor. LRP receptor consists of the non-covalently linked 515 kDa α-chain (Herz, J., *et al.*, *EMBO J.* 7:4119-4127 (1988)) containing binding sites for LRP ligands, and the 85 kDa transmembrane β-chain. Within the α-chain, cluster of cysteine-rich class A repeats is responsible for ligand binding (Moestrup, S. K., *et al.*, *J. Biol. Chem* 268:13691-13696 (1993)).

25   In contrast to the acidic ligand binding region in LRP, its ligands expose regions rich in positively charged amino acid residues (Moestrup, S.K., *Biochim. Biophys. Acta 1197*:197-213 (1994)). This type of binding and 31 class A repeats present in LRP may be responsible for its wide ligand diversity and ability to serve as a multi-ligand clearance receptor. LRP is expressed in many cell types and tissues

30   including placenta, lung and brain (Moestrup, S.K., *et al.*, *Cell Tissue Res.*

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269:375-382 (1992)) and is a major endocytic receptor in the liver (Strickland, D.K., *et al.*, *FASEB J.* 9:890-898 (1995)). A 39 kDa receptor-associated protein (RAP) binds to LRP with high affinity (K_d=4 nM (27)) and inhibits binding and LRP-mediated internalization and degradation of all ligands (Moestrup, S.K. *Biochim. Biophys. Acta* 1197:197-213 (1994); Williams, S.E., *et al.*, *J. Biol. Chem.* 267:9035-9040 (1992)), therefore serving as a useful tool for testing whether LRP is involved in endocytosis of a given ligand.

In the present study we demonstrated that fVIII specifically binds to LRP, and that LRP mediates the internalization and subsequent degradation of fVIII in cultured fibroblasts and appears to be responsible for *in vivo* clearance of fVIII from circulation. We also demonstrated that interaction of the A2 domain of fVIII with LRP is responsible for mediating catabolism of fVIII.

Experimental Procedures

Monoclonal Antibodies. The monoclonal antibodies (mAbs) C4 (epitope within the fVIII light chain residues 1670-1684 (Foster, P.A., *et al.*, *J. Biol. Chem* 263:5230-5234 (1988))), C5 (epitope within A1 residues 351-361) and T5 (epitope within the residues 701-740 (Fulcher, C.A., *et al.*, *J. Clin. Invest.* 76:117-124 (1985))) were kindly provided by Dr. Carol Fulcher (Scripps Clinic and Research Foundation, La Jolla, CA). The anti-A2 mAb 8860 was generously provided by Baxter/Hyland. Mab 413 (epitope within A2 domain residues 484-509 (Healey, *et al.*, J. F., *J. Biol. Chem* 270:14505-14509 (1995))) was prepared as described previously (Saenko, E.L., *et al.*, *J. Biol. Chem* 269:11601-11605 (1994)).

Proteins. LRP was isolated from human placenta as described (Ashcom, J.D., *et al.*, *J. Cell Biol.* 110:1041-1048 (1990)). Human RAP was expressed in bacteria and purified as described (Williams, S.E., *et al.*, *J. Biol. Chem.* 267:9035-9040 (1992)). FVIII was purified from therapeutic concentrates of

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Method M, American Red Cross (Saenko, E.L., *et al.*, *J. Biol. Chem* *271*:27424-27431 (1996)). HCh and LCh were prepared from fVIII as described previously (Saenko, E.L. and Scandella, D., *J. Biol Chem* 272, 18007-18014 (1995)). Purification of the A1/A3-C1-C2 dimer and A2 subunit was performed using ion exchange chromatography of thrombin activated fVIII on a Resource S column (Pharmacia) (Fay, P.T., *et al.*, *J. Biol. Chem* 268, 17861-17866 (1993)). Residual A2 present in the A1/A3-C1-C2 preparation was removed by its passage over an immobilized mAb 8860 column equilibrated in 20 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM $CaCl_2$.

Radiolabeling of fVIII and synthetic peptides. Prior to iodination fVIII and A2 were dialyzed into 0.2 M sodium acetate, 5 mM calcium nitrate, pH 6.8 (iodination buffer). Five μg of fVIII in 30 μl of iodination buffer were added to lactoperoxidase beads (Worthington Biochemical Corp.), 5 μl of $Na^{125}I$ (100 mCi/ml, Amersham), and 5 μl of 0.03% H_2O_2 (Mallincrodt) and incubated for 4 min. Free $Na^{125}I$ was removed by chromatography on a PD10 column (Pharmacia). The specific radioactivity of fVIII and A2 was 3.5-5 μCi/μg of protein. The activity of ^{125}I-fVIII determined in the one-stage clotting assay (3740 units/μg) was similar to that of unlabeled fVIII.

Solid-phase binding assays. Homologous and heterologous ligand displacement assays were performed as previously described (Williams, S.E., *et al.*, *J. Biol. Chem.* *267*:9035-9040 (1992)). Microtiter wells were coated with purified LRP or BSA (3 μg/ml) in 50 mM Tr-is, 0. 15 M NaCl, pH 8.0, for 16 h and then blocked with 3 % BSA in TBS. Coated wells were incubated with ^{125}I-A2 or ^{125}I-fVIII in 20 mM Tris-buffered saline pH 7.4, containing 5 mM $CaCl_2$, 0.05 % Tween-20 in the presence or absence of unlabeled competitors for 1 h at 37°C. The radioactivity bound to the wells was counted using a γ-counter (Pharmacia). Affinity constants were derived from homologous and heterologous displacement

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data using the computer program LIGAND (Munson, PT and Rodbard, D. *Anal. Biochem. 107*:220-239 (1980)).

Cell-mediated ligand internalization and degradation assays. A normal mouse embryonic fibroblast line (MEF) and a mouse embryonic fibroblast cell line that is genetically deficient in LRP biosynthesis (PEA 13) were obtained from Dr. Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX) and maintained as described (Willnow, T.E. and Herz, J., *J. Cell Sci. 107*:719-726 (1994)). Cells were seeded at 1x10⁵ cells/well and allowed to grow for 24 h at 37°C, 5% CO_2. Cellular internalization and degradation assays were conducted as described previously (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 270*:9307-9312 (1995)). Internalization and degradation of the ¹²⁵I-labeled fVIII and A2 was measured after incubation for indicated time intervals at 37°C in 0.5 ml of Dulbecco's modified medium (Gibco BRL) containing 2% BSA. Internalization was defined as radioactivity that is resistant to release from cells by trypsin (50 µg/ml) and proteinase K (50 µg/ml) (Sigma) in a buffer containing 5 mM EDTA. This treatment was previously shown to release radioligand bound to cell surface (Kounnas, M.Z., *et al.*, *J. Biol. Chem. 270*:9307-9312 (1995)) and therefore the ligand remained associated with cells after this treatment was considered as internalized. Degradation was defined as radioactivity in the medium that is soluble in 10% trichloroacetic acid. The value of degradation was corrected for non-cellular mediated degradation by subtracting the amount of degradation products generated in parallel wells lacking cells.

Clearance of ¹²⁵I-A2 domain and ¹²⁵I-fVIII/vWf complex from mouse plasma. The complex of ¹²⁵I-labeled fVIII with vWf in the presence or absence of RAP (in a total volume 250 µl) was injected in a tail vein of BALB/C mice over a period of approximately 20 seconds. At selected time intervals following injection (1, 3, 6, and 18 min), blood (50 µl) was withdrawn from the orbital plexus into 10 µl of 100 mM EDTA, and the radioactivity of the aliquot was determined. The

percentage of ligand remaining in circulation was calculated considering radioactivity of the aliquot taken at 1 min after injection as 100%. The clearance of each preparation was examined in two mice and the results were averaged. At the end of experiment, animals were sacrificed, liver lobules and kidneys were excised and weighed, followed by measuring the radioactivity in these tissues.

Results

Factor VIII binds to LRP and its binding is prevented by RAP. The ability of fVIII to bind to LRP *in vitro* was examined in homologous displacement binding assay. In the assay, binding of ^{125}I-fVIII (1 nM) to purified LRP, but not to BSA-coated wells, was competed (> 90%) by excess of unlabeled fVIII (Fig. 5A). The quantitative data regarding fVIII interaction with LRP were derived from the homologous displacement of ^{125}I-fVIII by unlabeled fVIII, which was adequately described by a model containing a single class of fVIII binding sites with K_d of 116 nM. To elucidate whether fVIII in a complex with vWf is also able to bind to LRP, we tested the effect of vWf on ^{125}I-fVIII binding to immobilized LRP. In this experiment, ^{125}I-fVIII was preincubated with vWf for 30 min at 37°C to allow complex formation prior to its addition to LRP coated wells. As shown in Fig. 5A, ^{125}I-fVIII binding to LRP was not inhibited by vWf up to the concentration of 1000 nM, which is 20-fold higher than its concentration in plasma (50 mM (Vlot, A.J., *et al.*, *Blood 85*:3150-3157 (1995))). This indicates that the complex formation with vWf does not affect fVIII ability to bind to LRP.

RAP, the antagonist of LRP-ligand binding, completely inhibited the binding of [125] I-fVIII to LRP-coated wells with K_i of 2.5 nM (Fig. 5B), a value similar to the previously determined affinity (4 nM) of RAP for LRP (Strickland, D.K., *et al.*, *J. Biol. Chem. 265*:17401-17404 (1990)). Together, these results demonstrate specific fVIII binding to LRP.

The amino acid residues 484-509 within the fVIII A2 domain are responsible for fVIII binding to purified LRP. In order to localize fVIII region(s) involved in interaction with LRP, binding between ^{125}I-fVIII and immobilized LRP was competed by unlabeled fVIII fragments. As shown in Fig. 6, HCh and A2 domain

5   of fVIII, but not LCh (AR-A3-C1-C2) or A1/A3-C1-C2 dimer, displaced ^{125}I-fVIII from LRP in the heterologous ligand displacement assay. The K_i values determined for the HCh and A2 were similar, 120 nM and 132 nM, respectively. The similarity of the above K_d value for fVIII binding to LRP and the K_i value for inhibition of this binding by isolated A2 subunit indicates that A2 domain of HCh

10   is responsible for fVIII binding to LRP.

To localize the region of the A2 domain responsible for the interaction with LRP, we tested the effect of anti-A2 monoclonal antibodies with known epitopes on fVIII/LRP binding. Fig. 7A shows that mAb 413 (epitope within the A2 domain residues 484-509 (Healey, J.F., et al., J. Biol. Chem 270:14505-14509

15   (1995))) but not mAb T5 (epitope within the A2 domain residues 701-740 (35)) is able to block fVIII/LRP interaction. The concentration of mAb 413 required for 50% inhibition of ^{125}I-fVIII/LRP binding was 2.5 nM. The low molar excess (2.5-fold) of mAb 413 over fVIII required for 50% inhibition of fVIII/LRP binding is consistent with a previously reported high affinity of mAb 413 for fVIII

20   (Lollar, P., et al., J. Clin. Invest. 93:2497-2504 (1994)). In a control experiment, mAbs C5 (epitope within A1 residues 351-361) and C4 (epitope within LCh residues 1670-1684 (Foster, P.A., et al., J. Biol. Chem 263:5230-5234 (1988))) did not have any effect on fVIII binding to LRP (data not shown), which is consistent with the lack of participation of A1 and LCh in fVIII binding to LRP.

25   Since it was previously demonstrated that mAb 413 recognizes synthetic peptide with a human fVIII sequence 484-509 (Healey, J.F., et al., J. Biol. Chem 270:14505-14509 (1995)), we tested if the region of the A2 domain encompassed by peptide 484-509 is involved in binding to LRP. As seen from Fig. 7B, the synthetic peptide 484-509, but not the control A2 peptide 432-456, inhibited fVIII

30   binding to LRP in a dose-dependent fashion, indicating that the region 484-509

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of the A2 domain contains critical residues for fVIII binding to LRP. In a control experiment, no binding of ^{125}I-fVIII to BSA-coated wells was observed in the presence of peptide 484-509 (Fig. 7B).

5

Internalization and degradation of ^{125}I-fVIII complex with vWf by cultured fibroblasts is mediated by LRP. Since the data presented above demonstrated specific interaction between fVIII and LRP, and vWf does not interfere with this interaction, we hypothesized that LRP may be also capable of mediating the cellular internalization of ^{125}I-fVIII from its complex with vWf. To examine this hypothesis, cellular uptake and degradation experiments were conducted in mouse

10   embryonal fibroblasts (MEF) which express LRP and in PEA 13 fibroblasts that are genetically deficient in LRP (Willnow, T.E. and Herz, J. *J. Cell Sci. 107*:719-726 (1994)). The ^{125}I-fVIII/vWf complex was prepared by 30 min (37°C) incubation of ^{125}I-fVIII with vWf at their plasma concentrations of 1 nM and 50 nM, respectively. As shown in Figs. 8A and B, MEF cells, but not PEA 13 cells

15   lacking LRP, were capable of internalizing and degrading of ^{125}I-fVIII in the presence of vWf. Further, internalization and degradation of ^{125}I-fVIII by MEF but not by PEA 13 fibroblasts was inhibited by RAP, an antagonist of ligand binding to LRP. The ability of RAP to block the uptake and degradation of ^{125}I-fVIII/vWf in MEF cells and inability of PEA 13 cells to efficiently mediate

20   uptake and degradation indicates that LRP is the mediator of ^{125}I-fVIII/vWf catabolism. To further characterize the degradation pathway of fVIII in the MEF cells, we tested the effect of chloroquine (an agent that blocks lysosomal degradation) on ^{125}I-fVIII degradation. As seen from Fig. 8B, the degradation of ^{125}I-fVIII is completely inhibited by chloroquine.

25   To elucidate if fVIII internalization in the absence of vWf is also mediated by LRP, we measured the internalization and degradation of isolated ^{125}I-fVIII (Fig. 9). As seen from Figs. 9A and B, both internalization and degradation of isolated ^{125}I-fVIII by MEF fibroblasts is approximately 2-fold higher than that in the presence of vWf. RAP inhibited internalization and degradation of ^{125}I-fVIII

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to a lesser degree than those of ^{125}I-fVIII/vWf complex and, in addition, LRP-
deficient PEA 13 fibroblasts were able to internalize and degrade isolated^{125}I-
fVIII. This indicates that LRP-mediated pathway is not the sole mechanism of
fVIII internalization and degradation in the absence of vWf.

To determine whether vWf bound to fVIII is also internalized and
degraded by MEF cells, internalization and degradation of ^{125}I-labeled vWf
complexed with fVIII was measured. As shown in Figs. 9A and B, the amounts
of internalized and degraded ^{125}I-vWf by both MEF and PEA 13 cells were less
than 5 % of the corresponding amounts of ^{125}I-fVIII catabolized from its complex
with vWf under the same experimental conditions. This indicates that vWf does
not follow fVIII in the LRP-mediated pathway and possibly dissociates from fVIII
at early stage of endocytosis, prior to entry of the complex into endosomal
compartments.

**The A2 subunit of fVIII inhibits endocytosis and degradation of ^{125}I-
fVIII/vWf by MEF cells.** Since we have demonstrated above that the A2 subunit
of fVIII prevents an *in vitro* interaction between LRP and fVIII, we examined if
A2 can also inhibit LRP-mediated internalization and degradation of fVIII/vWf
complex by MEF cells. Figs. 10A and B demonstrate that 1000-fold excess of the
A2 subunit over ^{125}I-fVIII/vWf complex effectively inhibit internalization (by
>70% after 4 hours) and degradation (by >60% after 4 hours) of this complex.
In contrast, A1/A3-C1-C2 heterodimer, which did not inhibit fVIII interaction with
purified LRP in the above experiments, did not have any effect on ^{125}I-fVIII
endocytosis and degradation by MEF cells (Fig. 10).

To confirm that the inhibitory effect of the A2 subunit results from its
direct competition with ^{125}I-fVIII/vWf complex for LRP-mediated internalization
and degradation, we tested whether MEF cells are able to internalize and degrade
isolated A2 subunit. As shown in Figs. 11A and B, ^{125}I-A2 is readily internalized
and degraded by LRP-expressing MEF cells. Both the internalization and
degradation of the ^{125}I-labeled A2 were blocked in the presence of RAP. In

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contrast, LRP-deficient PEA 13 cells were unable to internalize or degrade ^{125}I-A2 (Fig. 11), confirming that catabolism of the A2 subunit is LRP-mediated.

To verify that LRP-mediated internalization and degradation of the A2 domain was not the unique feature of the MEF cells, we tested ^{125}I-labeled A2 internalization and degradation by smooth muscle cells (SMC) and alveolar epithelial cells (T2), which also express LRP on their surfaces (Moestrup, S.K., *Cell Tissue Res.* 269:375-382 (1992)). As shown in Figs. 11C and D, RAP effectively inhibited both internalization of ^{125}I-A2 by SMC and T2 (by 81 % and 64 %, respectively), and its degradation (by 78 % and 68 %), indicating that these processes were mediated by LRP.

Thus, the data shown in Figs. 10 and 11 demonstrate that LRP is capable of binding fVIII via its A2 domain and of mediating fVIII endocytosis leading to lysosomal degradation.

Effect of RAP on the plasma clearance of ^{125}I-fVIII and ^{125}I-A2. To determine whether LRP is capable of catabolizing the isolated fVIII A2 subunit and whole fVIII from its complex with vWf *in vivo*, the effect of RAP on the clearance rates of ^{125}I-fVIII/vWf complex and ^{125}I-A2 in mice was tested. As shown in Fig. 12A, RAP increased the half-life of both ^{125}I-A2 and ^{125}I-fVIII in mouse plasma by approximately 4 and 2.5-fold, respectively. In addition, in the absence of RAP, most of radioactivity was found in the liver but not in kidney, consistent with LRP

presence in high abundance in hepatic tissues (Strickland, D.K., *et al.*, *FASEB J.* 9:890-898 (1995)). Thus, our data indicate that a RAP-sensitive hepatic receptor, LRP, plays a major role in the removal of fVIII and its A2 subunit from circulation.

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Discussion

In the present study we demonstrated that LRP mediates the internalization and degradation of human fVIII in a model system using LRP-expressing cells and is responsible for fVIII clearance *in vivo*. This conclusion is based on several independent observations. First of all, we found that fVIII directly binds to purified LRP immobilized on microtiter wells, and that this binding is competed by RAP, an antagonist of ligands binding to LRP. Second, ^{125}I-fVIII is internalized from its complex with vWf by mouse fibroblasts expressing LRP (MEF cells), but not by mouse fibroblasts genetically deficient in LRP (PEA 13 cells). Third, we demonstrated that RAP effectively inhibited the cellular uptake and degradation of ^{125}I-fVIII from its complex with vWf by MEF cells and *in vivo* clearance of ^{125}I-fVIII from circulation in mice.

Our studies revealed that the A2 domain of fVIII is responsible for its interaction with LRP, since only A2 domain and HCh, which contains the A2 domain, were able to inhibit the interaction of ^{125}I-fVIII with LRP in a purified system. Thus, it was concluded that A2 is responsible for fVIII binding to LRP. Based on the observation that vWf did not inhibit fVIII binding to LRP, we proposed that LRP may internalize fVIII from its complex with vWf. Indeed, mouse embryonic fibroblasts (MEF) that express LRP, but not fibroblasts genetically deficient in LRP, were able to internalize and degrade ^{125}I-fVIII in the presence of vWf. These processes were competed by RAP and A2 subunit of fVIII, indicating that cellular internalization and degradation were mediated by interaction of the A2 domain of fVIII with LRP. The physiological relevance of the observations utilizing the LRP-expressing cell model system was supported by *in vivo* clearance studies of ^{125}I-fVIII/vWf complex in mice which demonstrated that RAP prolonged the half-life of ^{125}I-fVIII in circulation by 2.5-fold, indicating that a RAP-sensitive receptor, most likely LRP, is responsible for the clearance of fVIII from plasma.

Further localization of the region within the A2 domain responsible for its binding to purified LRP was initiated by the finding that monoclonal antibody with an epitope within A2 domain residues 484-509 completely inhibited fVIII interaction with LRP. Inhibition of fVIII/LRP binding by synthetic peptide with a human fVIII sequence 484-509 indicated that the region of the A2 domain is likely to be directly involved in fVIII binding to purified LRP.

The region 484-509 contains 6 positively charged residues, Lys at positions 493, 496 and 499 and Arg at positions 484, 489 and 490. Basic residues in lipoprotein lipase (Chappell, D.A., et al., J. Biol. Chem. 268:14168-14175 (1993)), u-PA-PAI-1 complex (Rodenburg, K.W., et al., Biochem. J. 329:55-63 (1998)), and α_2-macroglobulin (Howard, G. C., et al., J. Biol. Chem 271:14105-14111 (1996)) were previously shown to be critical for electrostatic interaction with LRP. Alanine substitution of the basic amino acid residues in lipoprotein lipase (Williams, S.E., et al., J. Biol. Chem. 269:8653-8658 (1994)), u-PA/PAI-I complex (Rodenburg, K.W., et al., Biochem. J. 329:55-63 (1998)) and in the receptor binding fragment from α_2-macroglobulin (Howard, G.C., et al., J. Biol. Chem 271:14105-14111 (1996)) lead to a considerable reduction of affinity for ligand binding to LRP and partial (Rodenburg, K.W., et al., Biochem. J. 329:55-63 (1998)) or complete (Howard, G.C., et al., J. Biol. Chem 271:14105-14111 (1996)) inhibition of internalization and degradation of the mutants. Therefore, Ala or other amino acid substitutions within the 484-509 region of the recombinant fVIII are useful for reduction of the rate of its LRP-mediated endocytosis and generation of the fVIII mutants with a longer life in the circulation.

FVIII binds to purified LRP with affinity 116 nM, which is much lower than the concentration of fVIII/vWf complex in plasma (1 nM; Wion, K., et al., Nature 317:726-730 (1985)). FVIII affinity for LRP is similar to that of the complexes of serine proteases with inhibitors such as ATIII/thrombin (Kounnas, M.Z., et al., J. Biol. Chem. 271:6523-6529 (1996)), HCII/thrombin and α_1-antitrypsin/trypsin (Kounnas, M.Z., et al., J. Biol. Chem. 271:6523-6529 (1996)),

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which also bind to LRP with affinities 80-120 nM, and weaker than measured for other LRP ligands. It was shown (Kounnas, M.Z., *et al.*, *J. Biol. Chem.* *271*:6523-6529 (1996)) that internalization and degradation of the above low affinity LRP ligands at their 1 nM concentration by MEF cells occur at a lower

5   rate than that of the u-PA/PAI- I complex which binds to LRP with high affinity ($K_d < 1$ nM). Therefore, relatively low affinity of fVIII for LRP is responsible for a slow rate of fVIII internalization and degradation by MEF cells, which is comparable to the rate of ATIII/thrombin, HCII/thrombin and $\alpha 1$-antitrypsin/trypsin degradation at 1 nM concentration of each ligand. The low

10   affinity of fVIII for LRP may also be a necessary requirement for the relatively long fVIII half-life (12-14 h) in plasma of normal individuals (Over, J., *et al.*, *J. Clin. Invest.* *62*:223-234 (1978)). Alternatively, the low fVIII affinity for LRP may be compensated by concentration of fVIII molecules on the membrane of LRP-expressing cells, for example, via interaction with cell-surface proteoglycans

15   which have been shown to facilitate the uptake of a number of LRP ligands including lipoprotein lipase (Chappell, D.A., *et al.*, *J. Biol. Chem.* *268*:14168-14175 (1993)), hepatic lipase (Kounnas, M.Z., *et al.*, *J. Biol. Chem.* *270*:9307-9312 (1995)), and thrombospondin (Mikhailenko, I., *et al.*, *J. Biol. Chem.* *270*:9543-9549 (1995); Mikhailenko, I., *et al.*, *J. Biol. Chem.* *272*:6784-6791

20   (1997)).

We found that internalization and degradation of isolated fVIII by MEF cells was greater than the corresponding processes for fVIII bound to vWf. In addition, catabolism of the isolated fVIII by MEF cells was only partially inhibited by RAP, indicating that LRP-mediated endocytosis of fVIII is not the

25   sole mechanism of fVIII clearance in the absence of vWf. Our data suggest that in the presence of vWf, which blocks C2 domain-mediated fVIII binding to phospholipid membranes (Saenko, E.L. and Scandella, D., *J. Biol. Chem* *270*:13826-13833 (1995)), fVIII binds only to LRP, whereas in the absence of vWf, fVIII binds both to LRP and to an unidentified cell membrane component.

30   The latter binding may lead to fVIII internalization via RAP-independent

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pathway, which may be mediated by unidentified receptor as it was previously proposed for hepatic lipase (Kounnas, M.Z., *et al.*, *J. Biol. Chem.* *270*:9307-9312 (1995)). Since we found that ^{125}I-vWf is not internalized by MEF cells, we propose the model for fVIII endocytosis where fVIII/vWf complex binds to LRP and then vWf dissociates from fVIII during the early stage of fVIII endocytosis, *i.e.* during formation of the coated pits. Since the half-life for the dissociation of fVIII/vWf complex is about 1 hour (Saenko, E.L. and Scandella, D., *J. Biol Chem* 272, 18007-18014 *(1995)*), vWf may delay LRP-mediated endocytosis of fVIII according to the proposed model.

Faster catabolism of fVIII in the absence of vWf is consistent with a demonstrated shorter half-life of fVIII in patients with severe von Willebrand disease (vWD) lacking plasma vWf than that in hemophilia A patients, who have normal levels of vWf (Morfini, M., *et al.*, *Thromb. Haemostas.* *70*:270-272 (1993); Lethagen, S., *et al.*, *Ann. Hematol.* *65*:253-259 (1992)). Moreover, the half-life of fVIII in vWD patients was prolonged by the presence of vWf in the infused fVIII preparation (Lethagen, S., *et al.*, *Ann. Hematol.* *65*:253-259 (1992)). The above observations were previously explained by vWf-mediated stabilization of fVIII by binding to vWf (Wise, R.J., *et al.*, *J. Biol. Chem.* *266*:21948-21955 (1991)) and via secondary vWf-mediated release of endogenous fVIII (Wise, R.J., *et al.*, *J. Biol. Chem.* *266*:21948-21955 (1991); Kaufman, R.J., *Mol. Cell. Biol.* *9*:1233-1242 (1989)). Our data suggest that in addition to the above effects, vWf may reduce the rate of fVIII clearance by preventing LRP-independent pathway and limiting fVIII clearance to LRP-mediated pathway.

The activity of the factor X activation complex (factor Xase), consisting of membrane-bound activated fVIIIa and factor IXa, can be down regulated by inactivation of fVIIIa. The latter occurs via proteolytic degradation of fVIII by activated protein C, factor Xa and factor IXa, and via spontaneous but reversible dissociation of the A2 subunit from fVIIIa heterotrimer (Fay, P. J. and Smudzin, T. M., *J. Biol. Chem* *267*:13246-13250 (1992)). Dissociation of the fVIIIa heterotrimer may be accelerated by LRP mediated internalization of the A2

domain, and therefore complement regulation of fVIIIa activity at the sites of
coagulation. This hypothesis is supported by availability of LRP at these sites,
since LRP is exposed on the surface of monocytes and macrophage (Moestrup,
S.K., *et al.*, *Exp. Cell. Res. 190*:195-203 (1990); Moestrup, S.K., *et al.*, *Cell
Tissue Res. 269*:375-382 (1992)) and upon vascular injury on fibroblasts and
smooth muscle cells (Moestrup, S.K., *et al.*, *Cell Tissue Res. 269*:375-382
(1992)). In addition, it was recently shown that isolated A2 but not isolated Al
and A3-C1-C2 subunits of activated fVIII is able to accelerate factor IXa-
catalyzed conversion of factor X by approximately 100-fold (Fay, P.J. and
Koshibu, K., *Blood* 92:353a (abstract) (1998)). Even though acceleration of the
factor X activation by A2 is only 1 % of that in the presence of heterotrimeric
activated fVIII (Al/A2/A3-C1-C2) (Fay, P.J. and Koshibu, K., *Blood* 92:353a
(abstract) (1998)), it is possible that LRP-mediated removal of the A2, dissociated
from fVIIIa bound to a phospholipid membrane at the site of coagulation, is
important to prevent activation of factor X not in the place of the coagulation
event.

In summary, the current study demonstrates that LRP can bind fVIII/vWf
complex and mediate uptake of fVIII from it. *In vivo* clearance studies
underscored the likelihood that LRP indeed functions to remove LRP from
plasma.

Example 3

**Experiments on the development of recombinant fVIII molecule with
extended lifetime in circulation.** Since recombinant fVIII products are widely
used for fVIII replacement therapy in hemophiliacs who have decreased or
nonfunctional fVIII, generation of mutant(s) with a prolonged lifetime is a
promising approach to increase the efficacy and reduce the cost of fVIII infusion
therapy. A 39 kDa receptor associated protein (RAP) binds reversibly to LRP and
inhibits the binding of other ligands and therefore serves as a useful tool for

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testing whether LRP is involved in endocytosis of a given ligand. We found that fVIII binding to LRP is inhibited by RAP, confirming the specificity of this interaction. Since von Willebrand factor (vWf), bound to fVIII in the circulation, does not inhibit fVIII binding to purified LRP, we proposed that removal of the fVIII/vWf complex from the circulation may also be LRP-mediated. This role of LRP was supported by our finding that the lifetime of human ^{125}I-fVIII/vWf complex in mice was 2.5-times prolonged in the presence of RAP.

Based on our finding that fVIII amino acids 484-509 were important for fVIII binding to LRP, these amino acids are also important for LRP-mediated endocytosis. To identify the key fVIII amino acids required for endocytosis, single residues 484-509 are mutated to Ala in the B- domain deleted fVIII (B(-) fVIII). Since the basic residues are commonly involved in ligand binding to LRP, six basic residues within 484-509 (3 Lys and 3 Arg) are mutated. U.S. Patent No. 55,859,204 discloses the substitution to Ala of three of these residues (Arg[484,] Lys[493] and Arg[490]); however the other 3 residues – Arg[490], Lys[496] and Lys[499] – were not substituted. Thus, these residues, individually and in combination, are mutated to Ala. In particular, each of three Arg and each of three Lys are mutated by pairs (this implies preparation of 9 additional fVIII Ala double-mutants).

It is then determined whether endocytosis of the vWf complexes with B(-) fVIII mutant(s) by LRP-expressing cells is reduced compared to that of wild-type B (-) fVIII/vWf. Some mutations result in a decreased rate of internalization and a longer *in vivo* half-life of the complex of the B- fVIII mutant with vWf in plasma of mice compared to that of wild type B- fVIII/vWf complex. The data of the *in vivo* experiments performed in normal and fVIII-deficient mice is mathematically analyzed using biphasic time-course clearance model and equations approximating interspecies scaling which allow to predict fVIII half-life in humans (*Toxicology and Applied Pharmacology* 136:75-78 (1996)).

Clearance of mutant fVIII in vWf-deficient mice which lack fVIII in circulation (a mouse model for severe von Willebrand disease is described in *Proc. Natl. Acad. Sci. USA* 95:9524-9529 (1998)) is also analyzed. These

experiments are aimed at determining mutant fVIII's prolonged half-life in the absence of vWf. Factor VIII interaction with endothelial cells is also analyzed, since this interaction leads to fVIII internalization. In experiments using fluorescent microscopy techniques we observed uptake of fVIII by endothelial cells. Since a fine equilibrium exists in circulation between fVIII bound to vWf and fVIII bound and internalized by endothelial cells, fVIII interaction with phospholipid endothelial cell membrane is an important factor influencing concentration of fVIII (and hence its half-life) in circulation following fVIII injection.

Therefore, individual amino acids within the previously localized fVIII phospholipid binding site (C2 domain region 2303-2332) which play a role in fVIII binding to vWf and to phospholipid are identified. We identify the amino acids playing a key role in fVIII binding to phospholipid, but not to vWf. The amino acids which participate in fVIII binding to vWf and to phospholipids are selected based on the following observations. The homology search between the C2 domain of fVIII and the corresponding region of the discoidin and a family of homologous proteins, containing the so called DS domain, has revealed the fVIII C2 domain sequences involved in the formation of β-structures. In addition, it has been shown that the synthetic fVIII peptide 2310-2320 in which residues 2310 and 2320 are covalently linked to reproduce the corresponding loop structure within the C2 domain, competes for fVIII binding with vWf or phospholipid. Therefore, residues within the 2311-2319 region are mutated to Ala, and other amino acids. Since fV, a fVIII homolog, does not bind to vWf, we mutate only five residues which are unique within the 2311-2319 region of fVIII. The mutants are tested for binding to vWf and phospholipid, which identifies the fVIII residues playing a key role in binding to these ligands.

Clearance of the fVIII mutants with reduced phospholipid binding was compared with that of wt-fVIII in normal and hemophilic mice to determine the contribution of the phospholipid-dependent fVIII clearance component to total fVIII clearance.

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The mutations within the C2 domain region 2310-2320 prove to be effective for extension of fVIII lifetime in circulation, so we generate mutant fVIII in which both the C2 domain mutation(s) (positions 2310-2320) and mutation(s) within the A2 (positions 484-509) are combined.

We test the designed extended lifetime fVIII for gene therapy purposes. The extended lifetime fVIII gene is inserted in a virus-based vector, and delivered into hemophilia A mice. The time course of the fVIII *in vivo* expression level is assessed as follows: the number of the gene copies per cell (hepatic), the gene transcription level, fVIII activity and the antigen level are determined. Since it was shown that high titer antibodies increase clearance of fVIII (*Br. J. Hematol.* *93*:688-693 (1996)), we examine the immune response against the extended lifetime fVIII. We also compare its half-life in circulation in hemophilia A mice which formed antibodies against wild type fVIII.

What Is Claimed Is:

1.   A mutant factor VIII comprising an amino acid substitution at two
or more positions in the A2 domain; wherein at least one of said amino acid
substitutions is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501
or 508; wherein the mutant factor VIII has reduced receptor-dependent clearance;
and wherein the mutant factor VIII has procoagulant activity.

2.   The mutant factor VIII of claim 1, which lacks the B domain.

3.   The mutant factor VIII of claim 2, comprising an amino acid
substitution at two or more of positions 484 to 509.

4.   The mutant factor VIII of claim 3, comprising an amino acid
substitution at two or more of positions 490, 496 or 499.

5.   The mutant factor VIII of claim 3, comprising an amino acid
substitution at one or more of positions 490, 496 or 499; and at one or more of
positions 484, 489 or 493.

6.   The mutant factor VIII of claim 5, comprising an amino acid
substitution at position 490; and at one or more of positions 484, 489 or 493.

7.   The mutant factor VIII of claim 5, comprising an amino acid
substitution at position 496; and at one or more of positions 484, 489 or 493.

8.   The mutant factor VIII of claim 5, comprising an amino acid
substitution at position 499; and at one or more of positions 484, 489 or 493.

9.   The mutant factor VIII of claim 2, comprising SEQ ID NO:5.

10.   A pharmaceutically acceptable composition comprising the mutant factor VIII of claim 2.

11.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the mutant factor VIII of claim 2.

12.   The method of claim 11, which further comprises administering an effective amount of receptor associated protein (RAP).

13.   A polynucleotide encoding the mutant factor VIII of claim 2.

14.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polynucleotide of claim 13.

15.   The method of claim 14, which further comprises administering an effective amount of a polynucleotide encoding RAP.

16.   A mutant factor VIII comprising an amino acid substitution at one or more positions in the A2 domain, which is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; wherein the mutant factor VIII has reduced receptor-dependent clearance; and wherein the mutant factor VIII has procoagulant activity.

17.   The mutant factor VIII of claim 16, which lacks the B domain.

18.   The mutant factor VIII of claim 17, comprising an amino acid substitution at one or more of positions 484 to 509.

-65-

19.   The mutant factor VIII of claim 18, comprising an amino acid substitution at one or more of positions 490, 496 or 499.

20.   The mutant factor VIII of claim 19, comprising an amino acid substitution at position 490.

5   21.   The mutant factor VIII of claim 19, comprising an amino acid substitution at position 496.

22.   The mutant factor VIII of claim 19, comprising an amino acid substitution at position 499.

23.   The mutant factor VIII of claim 17, comprising SEQ ID NO:5.

10   24.   A pharmaceutically acceptable composition comprising the mutant factor VIII of claim 17.

25.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the mutant factor VIII of claim 17.

15   26.   The method of claim 25, which further comprises administering an effective amount of RAP.

27.   A polynucleotide encoding the mutant factor VIII of claim 17.

28.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polynucleotide of claim 27.

29.   The method of claim 28, which further comprises administering an effective amount of a polynucleotide encoding RAP.

30.   A mutant factor VIII comprising an amino acid substitution at one or more positions in the C2 domain; wherein the mutant factor VIII has reduced receptor-independent clearance; and wherein the mutant factor VIII has procoagulant activity.

31.   The mutant factor VIII of claim 30, which lacks the B domain.

32.   The mutant factor VIII of claim 31, comprising an amino acid substitution at one or more of positions 2303 to 2332.

33.   The mutant factor VIII of claim 32, comprising an amino acid substitution at one or more of positions 2311 to 2319.

34.   The mutant factor VIII of claim 31, comprising SEQ ID NO:1.

35.   A pharmaceutically acceptable composition comprising the mutant factor VIII of claim 31.

36.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the mutant factor VIII of claim 31.

37.   The method of claim 36, which further comprises administering an effective amount of RAP.

38.   A polynucleotide encoding the mutant factor VIII of claim 31.

39.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polynucleotide of claim 38.

40.   The method of claim 39, which further comprises administering an effective amount of a polynucleotide encoding RAP.

5   41.   A mutant factor VIII comprising:

(i)   an amino acid substitution at two or more positions in the A2 domain; wherein at least one of said amino acid substitutions is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; and

(ii)   an amino acid substitution at one or more positions in the

10   C2 domain as numbered in SEQ ID NO:1;

wherein the mutant factor VIII has reduced clearance; and wherein the mutant factor VIII has procoagulant activity.

42.   The mutant factor VIII of claim 41, which lacks the B domain.

43.   The mutant factor VIII of claim 42, comprising SEQ ID NO:5.

15   44.   A pharmaceutically acceptable composition comprising the mutant factor VIII of claim 42.

45.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the mutant factor VIII of claim 42.

20   46.   The method of claim 45, which further comprises administering an effective amount of RAP.

47.   A polynucleotide encoding the mutant factor VIII of claim 42.

48.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polynucleotide of claim 47.

49.   The method of claim 48, which further comprises administering an effective amount of a polynucleotide encoding RAP.

5   50.   A mutant factor VIII comprising:
        (i)   an amino acid substitution at one or more positions in the A2 domain, which is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; and
        (ii)   an amino acid substitution at one or more positions in the
10   C2 domain as numbered in SEQ ID NO:1;
        wherein the mutant factor VIII has reduced clearance; and wherein the mutant factor VIII has procoagulant activity.

51.   The mutant factor VIII of claim 50, which lacks the B domain.

52.   The mutant factor VIII of claim 51, comprising SEQ ID NO:5.

15   53.   A pharmaceutically acceptable composition comprising the mutant factor VIII of claim 51.

54.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the mutant factor VIII of claim 51.

20   55.   The method of claim 54, which further comprises administering an effective amount of RAP.

56.   A polynucleotide encoding the mutant factor VIII of claim 51.

57.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polynucleotide of claim 56.

58.   The method of claim 57, which further comprises administering an effective amount of a polynucleotide encoding RAP.

5   59.   A polypeptide selected from the group consisting of:

(a)   a polypeptide comprising a fragment of receptor-associated protein (RAP) which binds LRP;

(b)   a polypeptide comprising a mutant of RAP which binds LRP;

10   (c)   a polypeptide comprising an analog of RAP which binds LRP;

(d)   a polypeptide comprising 20 contiguous amino acids of the sequence of SEQ ID NO:4, which binds LRP; and

(e)   a polypeptide comprising amino acids 203 to 319 of SEQ
15   ID NO:4.

60.   A pharmaceutically acceptable composition comprising the polypeptide of claim 59.

61.   A method of treating hemophilia which comprises administering to a patient in need thereof an effective amount of the polypeptide of claim 59.

20   62.   The method of claim 61, which further comprises administering a mutant factor VIII having an amino acid substitution at one or more positions in the A2 domain.

63.   The method of claim 61, which further comprises administering a mutant factor VIII having an amino acid substitution at one or more positions in the C2 domain.

64.   The method of claim 61, which further comprises administering a mutant factor VIII having an amino acid substitution at one or more positions in the A2 domain and an amino acid substitution at one or more positions in the C2 domain.

65.   A method of increasing the half-life of factor VIII, selected from the group consisting of:

        (a)   a method which comprises substituting an amino acid at two or more positions in the A2 domain; wherein at least one of said amino acid substitutions is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; wherein the resulting factor VIII has reduced receptor-dependent clearance; and wherein the resulting factor VIII has procoagulant activity;

        (b)   method which comprises substituting an amino acid at one or more positions in the A2 domain, which is not at any of positions 484, 485, 487, 488, 489, 492, 493, 495, 501 or 508; wherein the resulting factor VIII has reduced receptor-dependent clearance; and wherein the resulting factor VIII has procoagulant activity;

        (c)   a method which comprises substituting an amino acid at one or more positions in the C2 domain; wherein the resulting factor VIII has reduced receptor-independent clearance; and wherein the resulting factor VIII has procoagulant activity;

        (d)   a method which comprises administering to a patient in need thereof an effective amount of a fragment of RAP, wherein said fragment binds LRP; and

        (e)   a method comprising two or more of methods (a), (b), (c) or (d).

FIG. 1
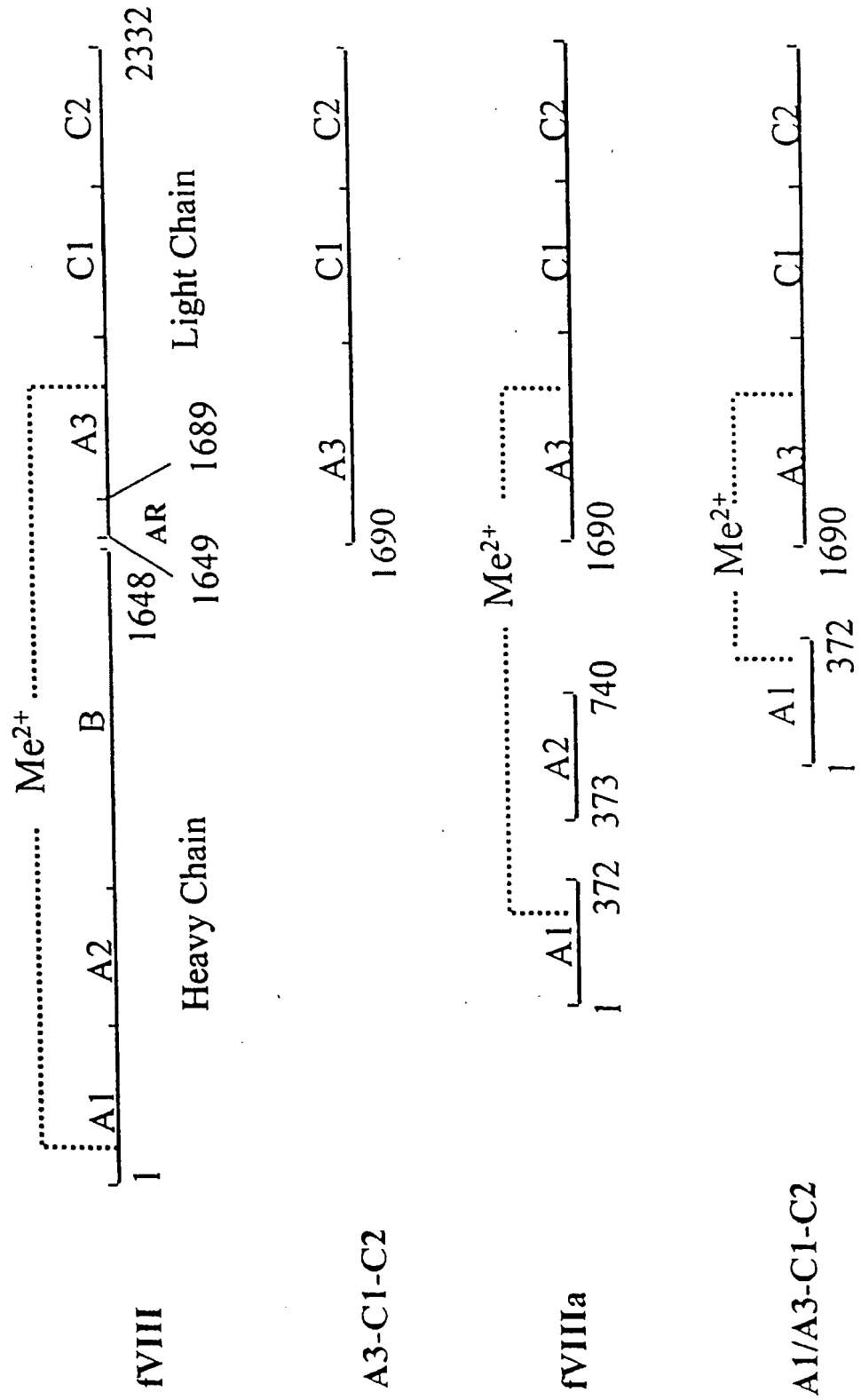
FIG. 2A

ATRRYYLGAVELSWDYMQSDLGELP

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VVITLKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQREKEDDKVFPGGSHTYVWQVL

KENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGSLAKEKTQTLHKFILLF

AVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGYVNRSLPGLIGCHRKSVYWHV

IGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLLMDLGQFLLFCHISSH

QHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNSPSFIQIR<u>S</u>

<u>VAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGRKYKKVRFMAY</u>

<u>TDETFKTREAIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRPLYSR</u>
<u>A2 Domain</u>   ******
                                                          LRP

<u>RLPKGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGL</u>
****
 Binding Region

<u>IGPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLED</u>

<u>PEFQASNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMV</u>

<u>YEDTLTLFPFSGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYED</u>

<u>SYEDISAYLLSKNNAIEPRE</u>ITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQS

PRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQ

FIG. 2B

PLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPR

KNFVKPNETKTYFWKVQHHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTN

TLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFH

AINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYN

LYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDF

QITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKF

SSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRL

HPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPSKAR

LHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQD

GHQWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLG

CEAQDLY

FIG. 3A

```
   1 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL GELPVDARFP PRVPKSFPFN

  61 TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY DTVVITLKNM ASHPVSLHAV

 121 GVSYWKASEG AEYDDQTSQR EKEDDKVFPG GSHTYVWQVL KENGPMASDP LCLTYSYLSH

 181 VDLVKDLNSG LIGALLVCRE GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD

 241 AASARAWPKM HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL EGHTFLVRNH

 301 RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME AYVKVDSCPE EPQLRMKNNE

 361 EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI RSVAKKHPKT WVHYIAAEEE DWDYAPLVLA

 421 PDDRSYKSQY LNNGPQRIGR KYKKVRFMAY TDETFKTREA IQHESGILGP LLYGEVGDTL

 481 LIIFKNQASR PYNIYPHGIT DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDGP

 541 TKSDPRCLTR YYSSFVNMER DLASGLIGPL LICYKESVDQ RGNQIMSDKR NVILFSVFDE

 601 NRSWYLTENI QRFLPNPAGV QLEDPEFQAS NIMHSINGYV FDSLQLSVCL HEVAYWYILS

 661 IGAQTDFLSV FFSGYTFKHK MVYEDTLTLF PFSGETVFMS MENPGLWILG CHNSDFRNRG

 721 MTALLKVSSC DKNTGDYYED SYEDISAYLL SKNNAIEPRS FSQNSRHRST RQKQFNATTI

 781 PENDIEKTDP WFAHRTPMPK IQNVSSSDLL MLLRQSPTPH GLSLSDLQEA KYETFSDDPS

 841 PGAIDSNNSL SEMTHFRPQL HHSGDMVFTP ESGLQLRLNE KLGTTAATEL KKLDFKVSST

 901 SNNLISTIPS DNLAAGTDNT SSLGPPSMPV HYDSQLDTTL FGKKSSPLTE SGGPLSLSEE

 961 NNDSKLLESG LMNSQESSWG KNVSSTESGR LFKGKRAHGP ALLTKDNALF KVSISLLKTN

1021 KTSNNSATNR KTHIDGPSLL IENSPSVWQN ILESDTEFKK VTPLIHDRML MDKNATALRL

1081 NHMSNKTTSS KNMEMVQQKK EGPIPPDAQN PDMSFFKMLF LPESARWIQR THGKNSLNSG

1141 QGPSPKQLVS LGPEKSVEGQ NFLSEKNKVV VGKGEFTKDV GLKEMVFPSS RNLFLTNLDN

1201 LHENNTHNQE KKIQEEIEKK ETLIQENVVL PQIHTVTGTK NFMKNLFLLS TRQNVEGSYD

1261 GAYAPVLQDF RSLNDSTNRT KKHTAHFSKK GEEENLEGLG NQTKQIVEKY ACTTRISPNT

1321 SQQNFVTQRS KRALKQFRLP LEETELEKRI IVDDTSTQWS KNMKHLTPST LTQIDYNEKE

1381 KGAITQSPLS DCLTRSHSIP QANRSPLPIA KVSSFPSIRP IYLTRVLFQD NSSHLPAASY

1441 RKKDSGVQES SHFLQGAKKN NLSLAILTLE MTGDQREVGS LGTSATNSVT YKKVENTVLP

1501 KPDLPKTSGK VELLPKVHIY QKDLFPTETS NGSPGHLDLV EGSLLQGTEG AIKWNEANRP

1561 GKVPFLRVAT ESSAKTPSKL LDPLAWDNHY GTQIPKEEWK SQEKSPEKTA FKKKDTILSL

1621 NACESNHAIA AINEGQNKPE IEVTWAKQGR TERLCSQNPP VLKRHQREIT RTTLQSDQEE
```

FIG. 3B

```
1681 IDYDDTISVE MKKEDFDIYD EDENQSPRSF QKKTRHYFIA AVERLWDYGM SSSPHVLRNR

1741 AQSGSVPQFK KVVFQEFTDG SFTQPLYRGE LNEHLGLLGP YIRAEVEDNI MVTFRNQASR

1801 PYSFYSSLIS YEEDQRQGAE PRKNFVKPNE TKTYFWKVQH HMAPTKDEFD CKAWAYFSDV

1861 DLEKDVHSGL IGPLLVCHTN TLNPAHGRQV TVQEFALFFT IFDETKSWYF TENMERNCRA

1921 PCNIQMEDPT FKENYRFHAI NGYIMDTLPG LVMAQDQRIR WYLLSMGSNE NIHSIHFSGH

1981 VFTVRKKEEY KMALYNLYPG VFETVEMLPS KAGIWRVECL IGEHLHAGMS TLFLVYSNKC

2041 QTPLGMASGH IRDFQITASG QYGQWAPKLA RLHYSGSINA WSTKEPFSWI KVDLLAPMII

2101 HGIKTQGARQ KFSSLYISQF IIMYSLDGKK WQTYRGNSTG TLMVFFGNVD SSGIKHNIFN

2161 PPIIARYIRL HPTHYSIRST LRMELMGCDL NSCSMPLGME SKAISDAQIT ASSYFTNMFA

2221 TWSPSKARLH LQGRSNAWRP QVNNPKEWLQ VDFQKTMKVT GVTTQGVKSL LTSMYVKEFL

2281 ISSSQDGHQW TLFFQNGKVK VFQGNQDSFT PVVNSLDPPL LTRYLRIHPQ SWVHQIALRM

2341 EVLGCEAQDL Y
```

FIG. 4

```
  1 MAPRRVRSFL RGLPALLLLL LFLGPWPAAS HGGKYSREKN QPKPSPKRES GEEFRMEKLN
    ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
              Signal Sequence


 61 QLWEKAQRLH LPPVRLAELH ADLKIQERDE LAWKKLKLDG LDEDGEKEAR LIRNLNVILA


121 KYGLDGKKDA RQVTSNSLSG TQEDGLDDPR LEKLWHKAKT SGKFSGEELD KLWREFLHHK


181 EKVHEYNVLL ETLSRTEEIH ENVISPSDLS DIKGSVLHSR HTELKEKLRS INQGLDRLRR
                                                                  * * * *


241 VSHQGYSTEA EFEEPRVIDL WDLAQSANLT DKELEAFREE LKHFEAKIEK HNHYQKQLEI
    * * * * * * * * * *   * * * * * * * * * *   * * * * * * * * * *   * * * * * * * * * *   * * * * * * * * * *   * * * * * * * * * *
                                  LDL Binding Region


301 AHEKLRHAES VGDGERVSRS REKHALLEGR TKELGYTVKK HLQDLSGRIS RARHNEL
    * * * * * * * * * *   * * * * * * * * * *   * * * * * * * * * *   * * * * * * * * * *   * * * * * * * * * *   * * *
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FIG. 5
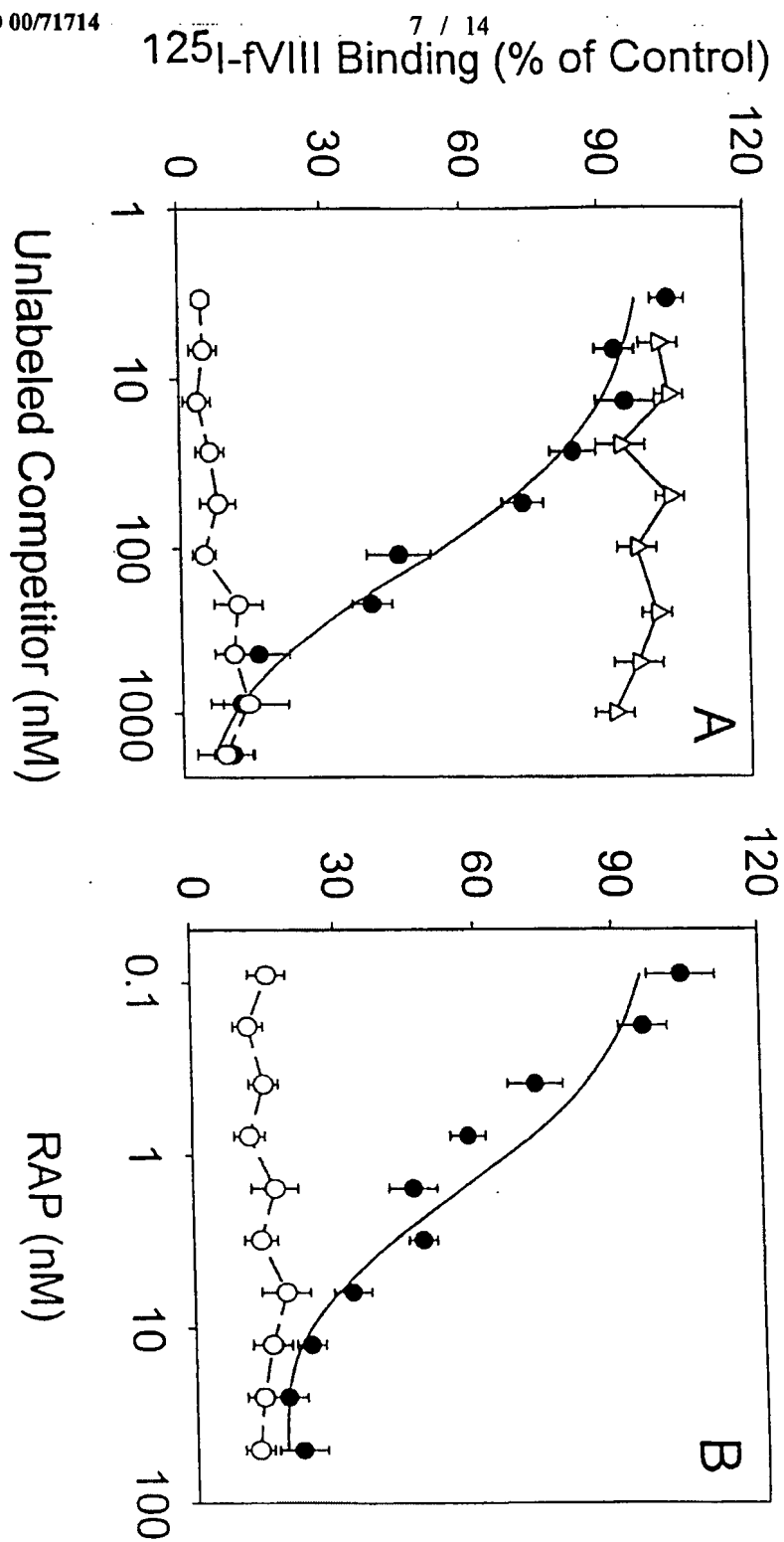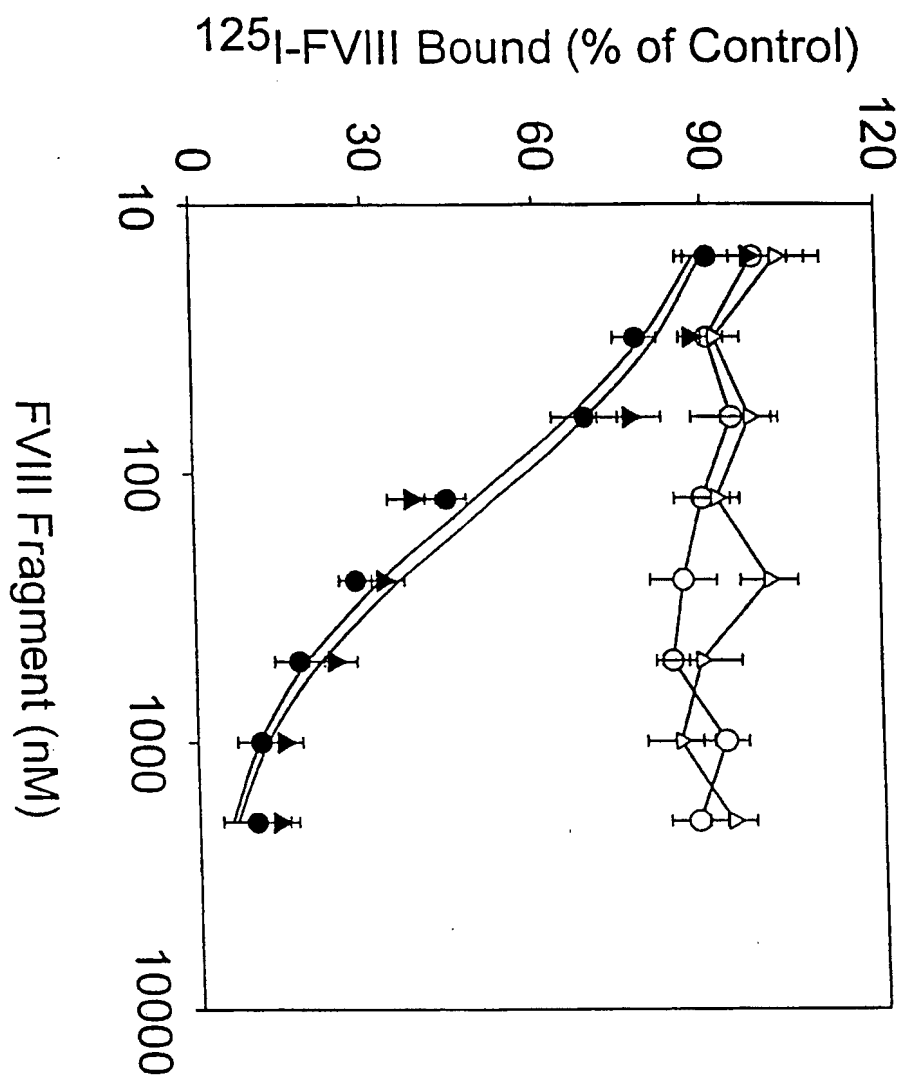
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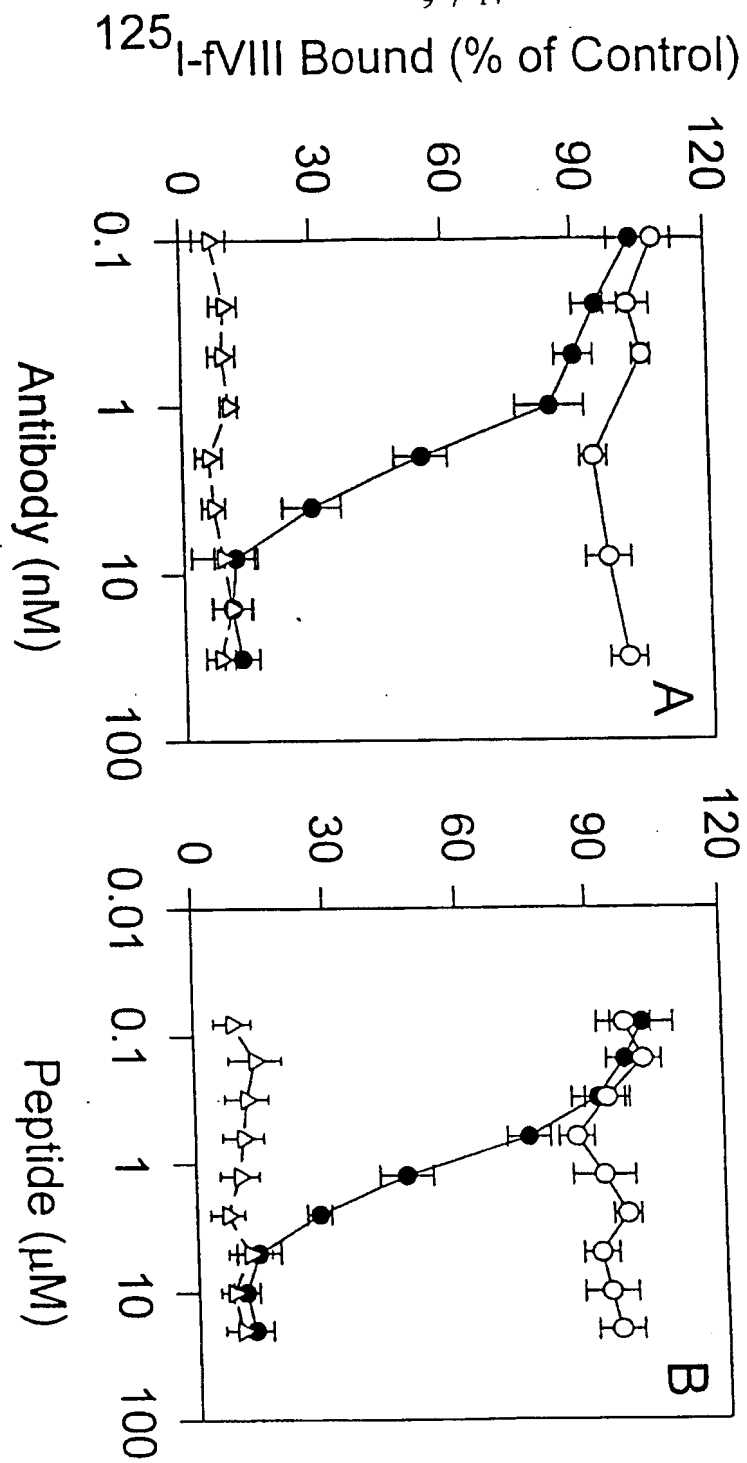
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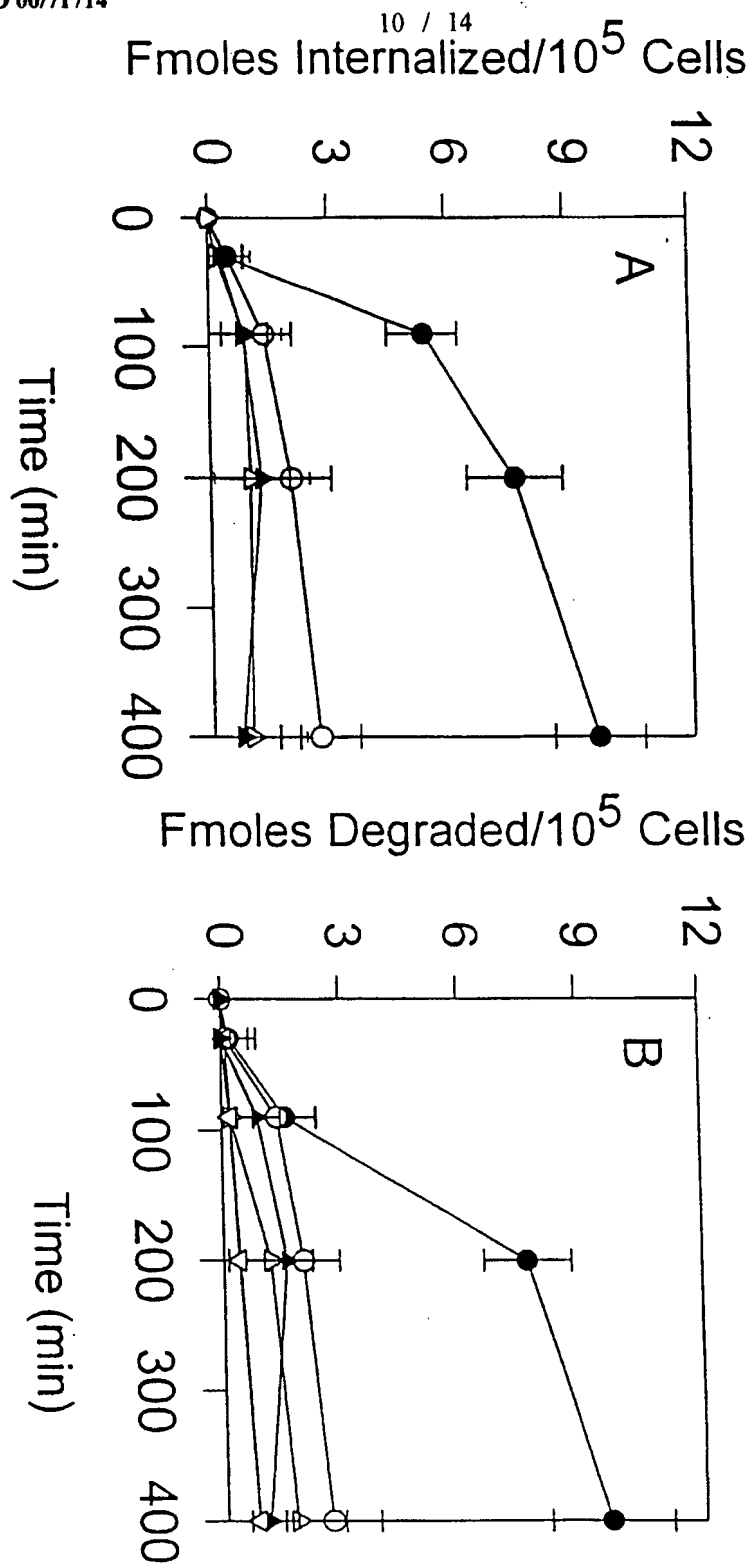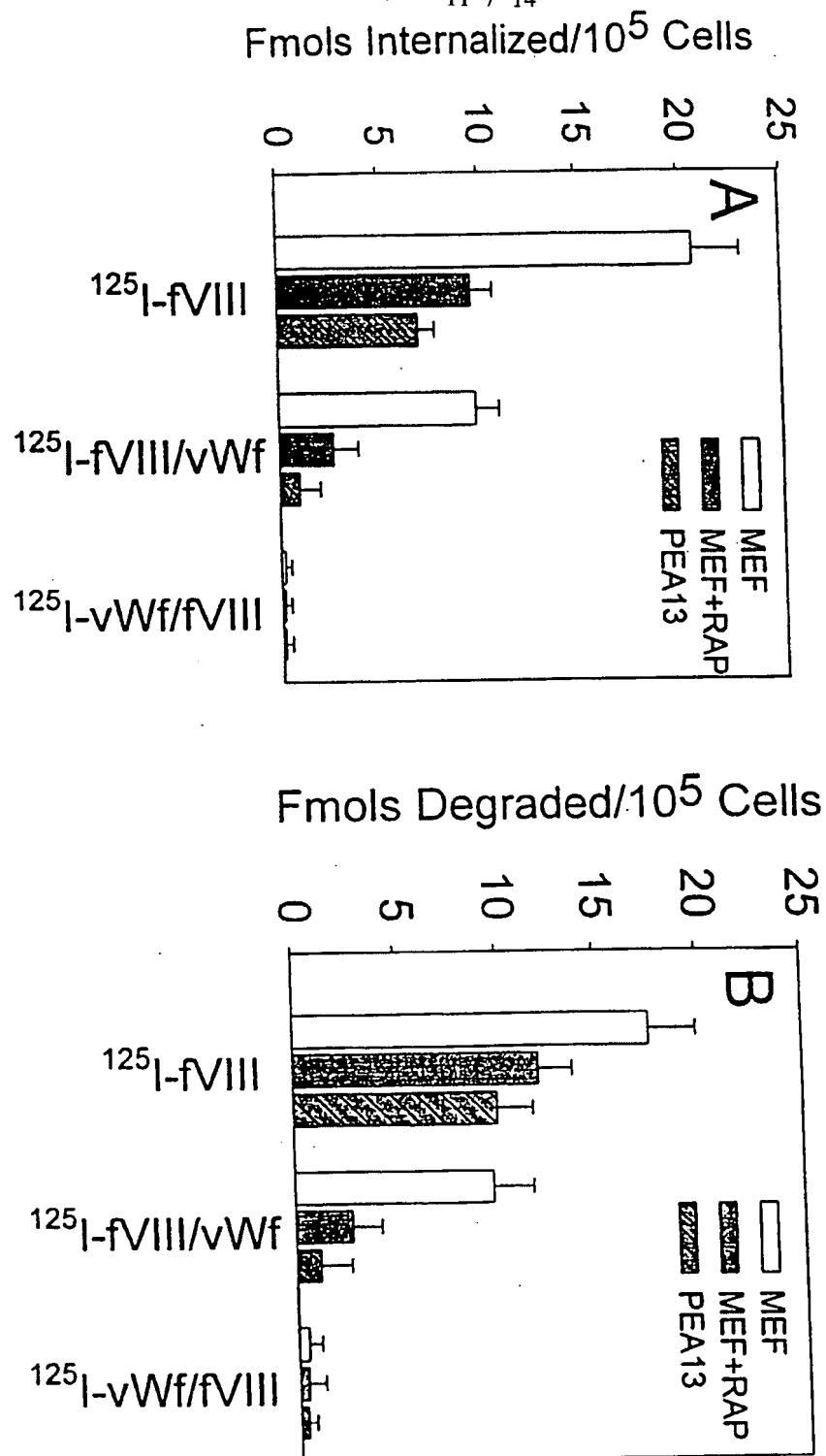
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A

Time (min)
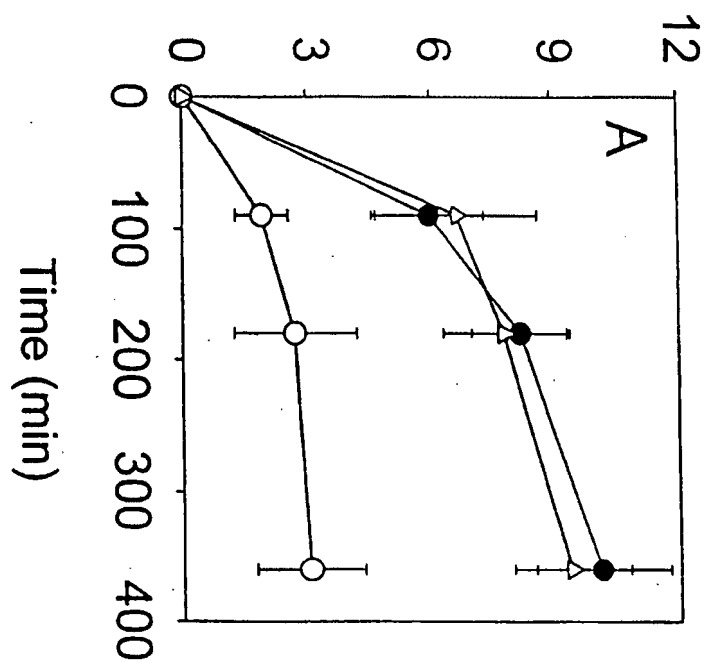
Fmoles Degraded/10^5 Cells

B

Time (min)

FIG. 8

FIG. 9

FIG. 10

FIG. 11
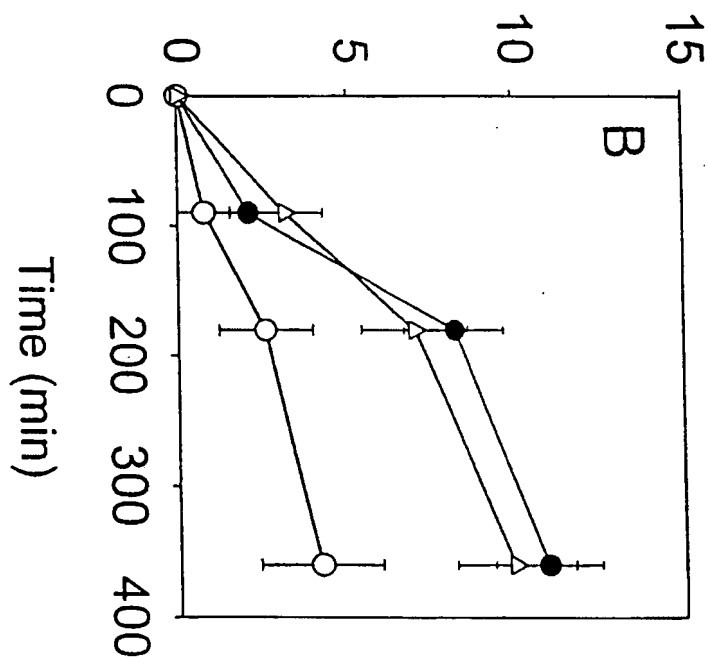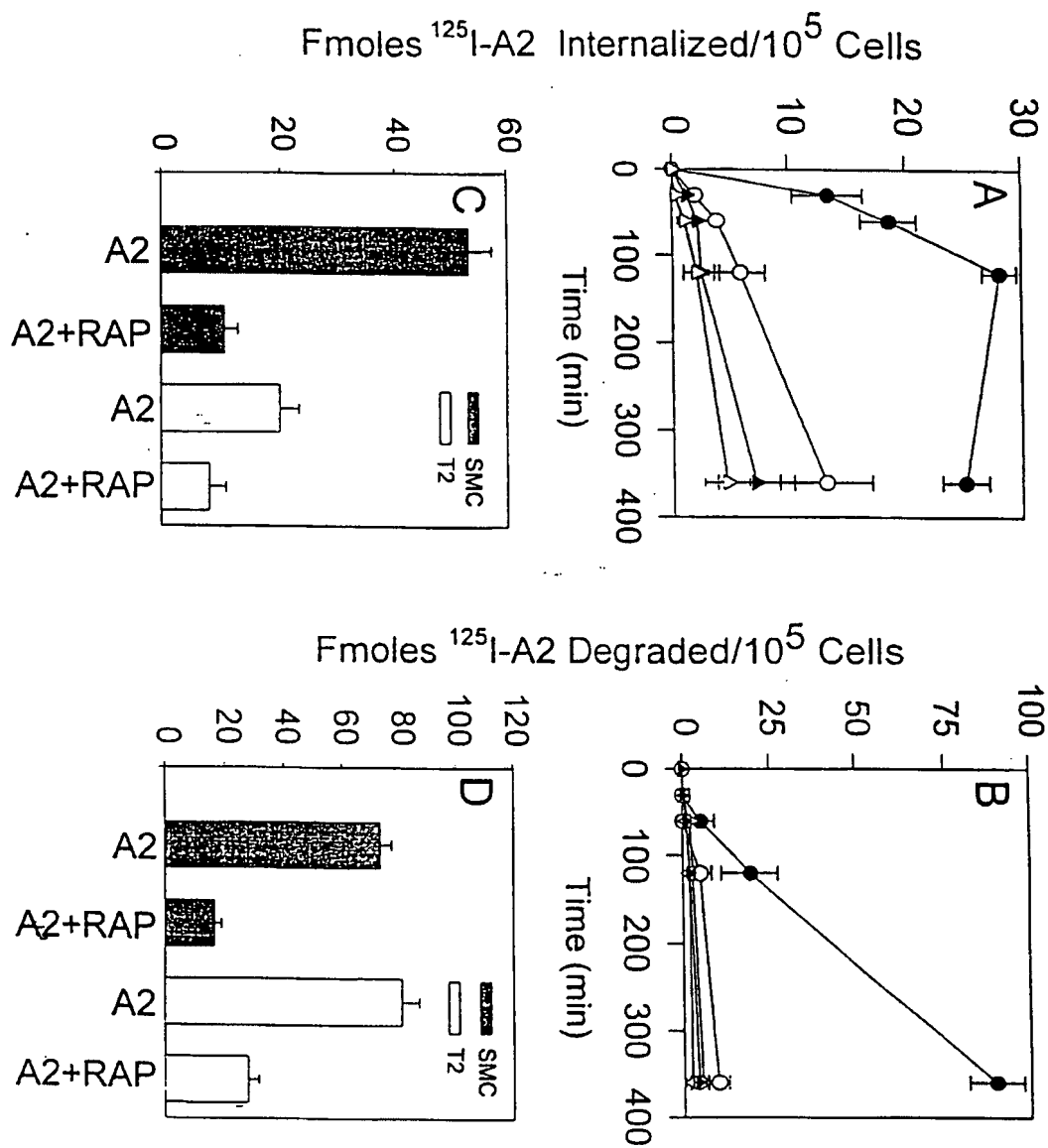
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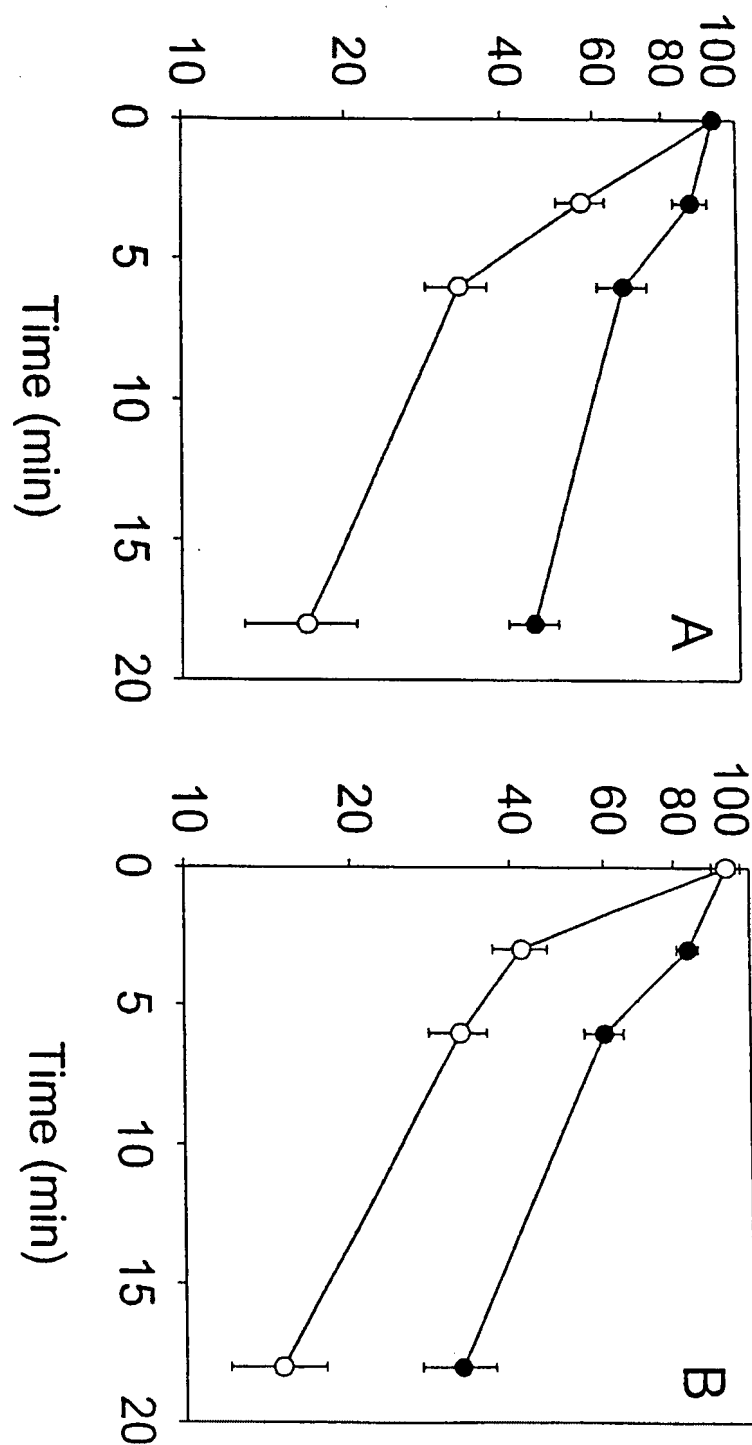
FIG. 12

-1-

SEQUENCE LISTING

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        Saenko, Evgueni L.
        Strickland, Dudley K.

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                                                        Met Gln Ile

-2-

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Glu Leu Ser Thr Cys Phe Phe Leu Cys Leu Leu Arg Phe Cys Phe Ser
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Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
 1           5                   10                  15

atg caa agt gat ctc ggt gag ctg cct gtg gac gca aga ttt cct cct   262
Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
             20                  25                  30

aga gtg cca aaa tct ttt cca ttc aac acc tca gtc gtg tac aaa aag   310
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         35                  40                  45

act ctg ttt gta gaa ttc acg gat cac ctt ttc aac atc gct aag cca   358
Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
     50                  55                  60

agg cca ccc tgg atg ggt ctg cta ggt cct acc atc cag gct gag gtt   406
Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
 65                  70                  75                  80

tat gat aca gtg gtc att aca ctt aag aac atg gct tcc cat cct gtc   454
Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
             85                  90                  95

agt ctt cat gct gtt ggt gta tcc tac tgg aaa gct tct gag gga gct   502
Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
             100                 105                 110

gaa tat gat gat cag acc agt caa agg gag aaa gaa gat gat aaa gtc   550
Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
             115                 120                 125

ttc cct ggt gga agc cat aca tat gtc tgg cag gtc ctg aaa gag aat   598
Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
     130                 135                 140

ggt cca atg gcc tct gac cca ctg tgc ctt acc tac tca tat ctt tct   646
Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145                 150                 155                 160

cat gtg gac ctg gta aaa gac ttg aat tca ggc ctc att gga gcc cta   694
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              His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
                           165                 170                 175


cta gta tgt aga gaa ggg agt ctg gcc aag gaa aag aca cag acc ttg   742
Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
             180                 185                 190


cac aaa ttt ata cta ctt ttt gct gta ttt gat gaa ggg aaa agt tgg   790
His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
         195                 200                 205


cac tca gaa aca aag aac tcc ttg atg cag gat agg gat gct gca tct   838
His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
     210                 215                 220


gct cgg gcc tgg cct aaa atg cac aca gtc aat ggt tat gta aac agg   886
Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225                 230                 235                 240


tct ctg cca ggt ctg att gga tgc cac agg aaa tca gtc tat tgg cat   934
Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
                 245                 250                 255


gtg att gga atg ggc acc act cct gaa gtg cac tca ata ttc ctc gaa   982
Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
                 260                 265                 270


ggt cac aca ttt ctt gtg agg aac cat cgc cag gcg tcc ttg gaa atc   1030
Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
             275                 280                 285


tcg cca ata act ttc ctt act gct caa aca ctc ttg atg gac ctt gga   1078
Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
         290                 295                 300


cag ttt cta ctg ttt tgt cat atc tct tcc cac caa cat gat ggc atg   1126
Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305                 310                 315                 320


gaa gct tat gtc aaa gta gac agc tgt cca gag gaa ccc caa cta cga   1174
Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
                 325                 330                 335


atg aaa aat aat gaa gaa gcg gaa gac tat gat gat gat ctt act gat   1222
Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
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                  340                    345                    350

tct gaa atg gat gtg gtc agg ttt gat gat gac aac tct cct tcc ttt    1270
Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
        355                    360                    365

atc caa att cgc tca gtt gcc aag aag cat cct aaa act tgg gta cat    1318
Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
        370                    375                    380

tac att gct gct gaa gag gag gac tgg gac tat gct ccc tta gtc ctc    1366
Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385                    390                    395                    400

gcc ccc gat gac aga agt tat aaa agt caa tat ttg aac aat ggc cct    1414
Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
                  405                    410                    415

cag cgg att ggt agg aag tac aaa aaa gtc cga ttt atg gca tac aca    1462
Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
                  420                    425                    430

gat gaa acc ttt aag act cgt gaa gct att cag cat gaa tca gga atc    1510
Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
        435                    440                    445

ttg gga cct tta ctt tat ggg gaa gtt gga gac aca ctg ttg att ata    1558
Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
        450                    455                    460

ttt aag aat caa gca agc aga cca tat aac atc tac cct cac gga atc    1606
Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465                    470                    475                    480

act gat gtc cgt cct ttg tat tca agg aga tta cca aaa ggt gta aaa    1654
Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
                  485                    490                    495

cat ttg aag gat ttt cca att ctg cca gga gaa ata ttc aaa tat aaa    1702
His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
                  500                    505                    510

tgg aca gtg act gta gaa gat ggg cca act aaa tca gat cct cgg tgc    1750
Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
                  515                    520                    525
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ctg acc cgc tat tac tct agt ttc gtt aat atg gag aga gat cta gct    1798
Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
    530             535             540

tca gga ctc att ggc cct ctc ctc atc tgc tac aaa gaa tct gta gat    1846
Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545             550             555             560

caa aga gga aac cag ata atg tca gac aag agg aat gtc atc ctg ttt    1894
Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
                565             570             575

tct gta ttt gat gag aac cga agc tgg tac ctc aca gag aat ata caa    1942
Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
                580             585             590

cgc ttt ctc ccc aat cca gct gga gtg cag ctt gag gat cca gag ttc    1990
Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
                595             600             605

caa gcc tcc aac atc atg cac agc atc aat ggc tat gtt ttt gat agt    2038
Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
    610             615             620

ttg cag ttg tca gtt tgt ttg cat gag gtg gca tac tgg tac att cta    2086
Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625             630             635             640

agc att gga gca cag act gac ttc ctt tct gtc ttc ttc tct gga tat    2134
Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
                645             650             655

acc ttc aaa cac aaa atg gtc tat gaa gac aca ctc acc cta ttc cca    2182
Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
            660             665             670

ttc tca gga gaa act gtc ttc atg tcg atg gaa aac cca ggt cta tgg    2230
Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
            675             680             685

att ctg ggg tgc cac aac tca gac ttt cgg aac aga ggc atg acc gcc    2278
Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
    690             695             700
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tta ctg aag gtt tct agt tgt gac aag aac act ggt gat tat tac gag    2326
Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705             710             715             720


gac agt tat gaa gat att tca gca tac ttg ctg agt aaa aac aat gcc    2374
Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
            725             730             735


att gaa cca aga agc ttc tcc cag aat tca aga cac cgt agc act agg    2422
Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg Ser Thr Arg
            740             745             750


caa aag caa ttt aat gcc acc aca att cca gaa aat gac ata gag aag    2470
Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
            755             760             765


act gac cct tgg ttt gca cac aga aca cct atg cct aaa ata caa aat    2518
Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
            770             775             780


gtc tcc tct agt gat ttg ttg atg ctc ttg cga cag agt cct act cca    2566
Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
785             790             795             800


cat ggg cta tcc tta tct gat ctc caa gaa gcc aaa tat gag act ttt    2614
His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
            805             810             815


tct gat gat cca tca cct gga gca ata gac agt aat aac agc ctg tct    2662
Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
            820             825             830


gaa atg aca cac ttc agg cca cag ctc cat cac agt ggg gac atg gta    2710
Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
            835             840             845


ttt acc cct gag tca ggc ctc caa tta aga tta aat gag aaa ctg ggg    2758
Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
            850             855             860


aca act gca gca aca gag ttg aag aaa ctt gat ttc aaa gtt tct agt    2806
Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser
865             870             875             880


aca tca aat aat ctg att tca aca att cca tca gac aat ttg gca gca    2854
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Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala
            885                 890                 895


ggt act gat aat aca agt tcc tta gga ccc cca agt atg cca gtt cat    2902
Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
            900                 905                 910


tat gat agt caa tta gat acc act cta ttt ggc aaa aag tca tct ccc    2950
Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
            915                 920                 925


ctt act gag tct ggt gga cct ctg agc ttg agt gaa gaa aat aat gat    2998
Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
            930                 935                 940


tca aag ttg tta gaa tca ggt tta atg aat agc caa gaa agt tca tgg    3046
Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp
945                 950                 955                 960


gga aaa aat gta tcg tca aca gag agt ggt agg tta ttt aaa ggg aaa    3094
Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
                965                 970                 975


aga gct cat gga cct gct ttg ttg act aaa gat aat gcc tta ttc aaa    3142
Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
            980                 985                 990


gtt agc atc tct ttg tta aag aca aac aaa act tcc aat aat tca gca    3190
Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala
            995                 1000                1005


act aat aga aag act cac att gat ggc cca tca tta tta att gag aat    3238
Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu Asn
    1010                1015                1020


agt cca tca gtc tgg caa aat ata tta gaa agt gac act gag ttt aaa    3286
Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu Phe Lys
1025                1030                1035                1040


aaa gtg aca cct ttg att cat gac aga atg ctt atg gac aaa aat gct    3334
Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp Lys Asn Ala
                1045                1050                1055


aca gct ttg agg cta aat cat atg tca aat aaa act act tca tca aaa    3382
Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr Thr Ser Ser Lys
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                1060              1065              1070


aac atg gaa atg gtc caa cag aaa aaa gag ggc ccc att cca cca gat   3430
Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly Pro Ile Pro Pro Asp
       1075              1080              1085


gca caa aat cca gat atg tcg ttc ttt aag atg cta ttc ttg cca gaa   3478
Ala Gln Asn Pro Asp Met Ser Phe Phe Lys Met Leu Phe Leu Pro Glu
       1090              1095              1100


tca gca agg tgg ata caa agg act cat gga aag aac tct ctg aac tct   3526
Ser Ala Arg Trp Ile Gln Arg Thr His Gly Lys Asn Ser Leu Asn Ser
1105              1110              1115              1120


ggg caa ggc ccc agt cca aag caa tta gta tcc tta gga cca gaa aaa   3574
Gly Gln Gly Pro Ser Pro Lys Gln Leu Val Ser Leu Gly Pro Glu Lys
              1125              1130              1135


tct gtg gaa ggt cag aat ttc ttg tct gag aaa aac aaa gtg gta gta   3622
Ser Val Glu Gly Gln Asn Phe Leu Ser Glu Lys Asn Lys Val Val Val
              1140              1145              1150


gga aag ggt gaa ttt aca aag gac gta gga ctc aaa gag atg gtt ttt   3670
Gly Lys Gly Glu Phe Thr Lys Asp Val Gly Leu Lys Glu Met Val Phe
              1155              1160              1165


cca agc agc aga aac cta ttt ctt act aac ttg gat aat tta cat gaa   3718
Pro Ser Ser Arg Asn Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu
       1170              1175              1180


aat aat aca cac aat caa gaa aaa aaa att cag gaa gaa ata gaa aag   3766
Asn Asn Thr His Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu Lys
1185              1190              1195              1200


aag gaa aca tta atc caa gag aat gta gtt ttg cct cag ata cat aca   3814
Lys Glu Thr Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr
              1205              1210              1215


gtg act ggc act aag aat ttc atg aag aac ctt ttc tta ctg agc act   3862
Val Thr Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr
              1220              1225              1230


agg caa aat gta gaa ggt tca tat gac ggg gca tat gct cca gta ctt   3910
Arg Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala Pro Val Leu
       1235              1240              1245
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caa gat ttt agg tca tta aat gat tca aca aat aga aca aag aaa cac    3958
Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys His
   1250            1255            1260


aca gct cat ttc tca aaa aaa ggg gag gaa gaa aac ttg gaa ggc ttg    4006
Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu Asn Leu Glu Gly Leu
1265            1270             1275            1280


gga aat caa acc aag caa att gta gag aaa tat gca tgc acc aca agg    4054
Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys Thr Thr Arg
            1285            1290            1295


ata tct cct aat aca agc cag cag aat ttt gtc acg caa cgt agt aag    4102
Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr Gln Arg Ser Lys
            1300            1305            1310


aga gct ttg aaa caa ttc aga ctc cca cta gaa gaa aca gaa ctt gaa    4150
Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu Glu Thr Glu Leu Glu
      1315            1320            1325


aaa agg ata att gtg gat gac acc tca acc cag tgg tcc aaa aac atg    4198
Lys Arg Ile Ile Val Asp Asp Thr Ser Thr Gln Trp Ser Lys Asn Met
   1330            1335            1340


aaa cat ttg acc ccg agc acc ctc aca cag ata gac tac aat gag aag    4246
Lys His Leu Thr Pro Ser Thr Leu Thr Gln Ile Asp Tyr Asn Glu Lys
1345            1350            1355            1360


gag aaa ggg gcc att act cag tct ccc tta tca gat tgc ctt acg agg    4294
Glu Lys Gly Ala Ile Thr Gln Ser Pro Leu Ser Asp Cys Leu Thr Arg
            1365            1370            1375


agt cat agc atc cct caa gca aat aga tct cca tta ccc att gca aag    4342
Ser His Ser Ile Pro Gln Ala Asn Arg Ser Pro Leu Pro Ile Ala Lys
            1380            1385            1390


gta tca tca ttt cca tct att aga cct ata tat ctg acc agg gtc cta    4390
Val Ser Ser Phe Pro Ser Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu
      1395            1400            1405


ttc caa gac aac tct tct cat ctt cca gca gca tct tat aga aag aaa    4438
Phe Gln Asp Asn Ser Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys
   1410            1415            1420
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gat tct ggg gtc caa gaa agc agt cat ttc tta caa gga gcc aaa aaa    4486
Asp Ser Gly Val Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys Lys
1425            1430            1435            1440


aat aac ctt tct tta gcc att cta acc ttg gag atg act ggt gat caa    4534
Asn Asn Leu Ser Leu Ala Ile Leu Thr Leu Glu Met Thr Gly Asp Gln
            1445            1450            1455


aga gag gtt ggc tcc ctg ggg aca agt gcc aca aat tca gtc aca tac    4582
Arg Glu Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser Val Thr Tyr
        1460            1465            1470


aag aaa gtt gag aac act gtt ctc ccg aaa cca gac ttg ccc aaa aca    4630
Lys Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu Pro Lys Thr
        1475            1480            1485


tct ggc aaa gtt gaa ttg ctt cca aaa gtt cac att tat cag aag gac    4678
Ser Gly Lys Val Glu Leu Leu Pro Lys Val His Ile Tyr Gln Lys Asp
    1490            1495            1500


cta ttc cct acg gaa act agc aat ggg tct cct ggc cat ctg gat ctc    4726
Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser Pro Gly His Leu Asp Leu
1505            1510            1515            1520


gtg gaa ggg agc ctt ctt cag gga aca gag gga gcg att aag tgg aat    4774
Val Glu Gly Ser Leu Leu Gln Gly Thr Glu Gly Ala Ile Lys Trp Asn
        1525            1530            1535


gaa gca aac aga cct gga aaa gtt ccc ttt ctg aga gta gca aca gaa    4822
Glu Ala Asn Arg Pro Gly Lys Val Pro Phe Leu Arg Val Ala Thr Glu
        1540            1545            1550


agc tct gca aag act ccc tcc aag cta ttg gat cct ctt gct tgg gat    4870
Ser Ser Ala Lys Thr Pro Ser Lys Leu Leu Asp Pro Leu Ala Trp Asp
        1555            1560            1565


aac cac tat ggt act cag ata cca aaa gaa gag tgg aaa tcc caa gag    4918
Asn His Tyr Gly Thr Gln Ile Pro Lys Glu Glu Trp Lys Ser Gln Glu
    1570            1575            1580


aag tca cca gaa aaa aca gct ttt aag aaa aag gat acc att ttg tcc    4966
Lys Ser Pro Glu Lys Thr Ala Phe Lys Lys Lys Asp Thr Ile Leu Ser
1585            1590            1595            1600


ctg aac gct tgt gaa agc aat cat gca ata gca gca ata aat gag gga    5014
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Leu Asn Ala Cys Glu Ser Asn His Ala Ile Ala Ala Ile Asn Glu Gly
         1605             1610            1615


caa aat aag ccc gaa ata gaa gtc acc tgg gca aag caa ggt agg act   5062
Gln Asn Lys Pro Glu Ile Glu Val Thr Trp Ala Lys Gln Gly Arg Thr
         1620             1625            1630


gaa agg ctg tgc tct caa aac cca cca gtc ttg aaa cgc cat caa cgg   5110
Glu Arg Leu Cys Ser Gln Asn Pro Pro Val Leu Lys Arg His Gln Arg
         1635             1640            1645


gaa ata act cgt act act ctt cag tca gat caa gag gaa att gac tat   5158
Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr
      1650             1655            1660


gat gat acc ata tca gtt gaa atg aag aag gaa gat ttt gac att tat   5206
Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr
1665             1670            1675             1680


gat gag gat gaa aat cag agc ccc cgc agc ttt caa aag aaa aca cga   5254
Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
         1685             1690            1695


cac tat ttt att gct gca gtg gag agg ctc tgg gat tat ggg atg agt   5302
His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser
         1700             1705            1710


agc tcc cca cat gtt cta aga aac agg gct cag agt ggc agt gtc cct   5350
Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro
         1715             1720            1725


cag ttc aag aaa gtt gtt ttc cag gaa ttt act gat ggc tcc ttt act   5398
Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr
      1730             1735            1740


cag ccc tta tac cgt gga gaa cta aat gaa cat ttg gga ctc ctg ggg   5446
Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu Leu Gly
1745             1750            1755             1760


cca tat ata aga gca gaa gtt gaa gat aat atc atg gta act ttc aga   5494
Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg
            1765             1770            1775


aat cag gcc tct cgt ccc tat tcc ttc tat tct agc ctt att tct tat   5542
Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr
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           1780              1785              1790

gag gaa gat cag agg caa gga gca gaa cct aga aaa aac ttt gtc aag    5590
Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys
        1795              1800              1805

cct aat gaa acc aaa act tac ttt tgg aaa gtg caa cat cat atg gca    5638
Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln His His Met Ala
    1810              1815              1820

ccc act aaa gat gag ttt gac tgc aaa gcc tgg gct tat ttc tct gat    5686
Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp
1825              1830              1835              1840

gtt gac ctg gaa aaa gat gtg cac tca ggc ctg att gga ccc ctt ctg    5734
Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu
            1845              1850              1855

gtc tgc cac act aac aca ctg aac cct gct cat ggg aga caa gtg aca    5782
Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr
            1860              1865              1870

gta cag gaa ttt gct ctg ttt ttc acc atc ttt gat gag acc aaa agc    5830
Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser
        1875              1880              1885

tgg tac ttc act gaa aat atg gaa aga aac tgc agg gct ccc tgc aat    5878
Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn
    1890              1895              1900

atc cag atg gaa gat ccc act ttt aaa gag aat tat cgc ttc cat gca    5926
Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala
1905              1910              1915              1920

atc aat ggc tac ata atg gat aca cta cct ggc tta gta atg gct cag    5974
Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln
            1925              1930              1935

gat caa agg att cga tgg tat ctg ctc agc atg ggc agc aat gaa aac    6022
Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn
            1940              1945              1950

atc cat tct att cat ttc agt gga cat gtg ttc act gta cga aaa aaa    6070
Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys
        1955              1960              1965
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gag gag tat aaa atg gca ctg tac aat ctc tat cca ggt gtt ttt gag    6118
Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu
    1970            1975            1980


aca gtg gaa atg tta cca tcc aaa gct gga att tgg cgg gtg gaa tgc    6166
Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys
1985            1990            1995            2000


ctt att ggc gag cat cta cat gct ggg atg agc aca ctt ttt ctg gtg    6214
Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val
            2005            2010            2015


tac agc aat aag tgt cag act ccc ctg gga atg gct tct gga cac att    6262
Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile
        2020            2025            2030


aga gat ttt cag att aca gct tca gga caa tat gga cag tgg gcc cca    6310
Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro
        2035            2040            2045


aag ctg gcc aga ctt cat tat tcc gga tca atc aat gcc tgg agc acc    6358
Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr
    2050            2055            2060


aag gag ccc ttt tct tgg atc aag gtg gat ctg ttg gca cca atg att    6406
Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile
2065            2070            2075            2080


att cac ggc atc aag acc cag ggt gcc cgt cag aag ttc tcc agc ctc    6454
Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu
            2085            2090            2095


tac atc tct cag ttt atc atc atg tat agt ctt gat ggg aag aag tgg    6502
Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp
            2100            2105            2110


cag act tat cga gga aat tcc act gga acc tta atg gtc ttc ttt ggc    6550
Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly
        2115            2120            2125


aat gtg gat tca tct ggg ata aaa cac aat att ttt aac cct cca att    6598
Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile
    2130            2135            2140
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att gct tga tac atc cgt ttg cac cca act cat tat agc att cgc agc     6646
Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser
2145            2150                2155                2160


act ctt cgc atg gag ttg atg ggc tgt gat tta aat agt tgc agc atg     6694
Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met
            2165                2170                2175


cca ttg gga atg gag agt aaa gca ata tca gat gca cag att act gct     6742
Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala
            2180                2185                2190


tca tcc tac ttt acc aat atg ttt gcc acc tgg tct cct tca aaa gct     6790
Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala
         2195                2200                2205


cga ctt cac ctc caa ggg agg agt aat gcc tgg aga cct cag gtg aat     6838
Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn
      2210                2215                2220


aat cca aaa gag tgg ctg caa gtg gac ttc cag aag aca atg aaa gtc     6886
Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met Lys Val
2225                2230                2235                2240


aca gga gta act act cag gga gta aaa tct ctg ctt acc agc atg tat     6934
Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr Ser Met Tyr
            2245                2250                2255


gtg aag gag ttc ctc atc tcc agc agt caa gat ggc cat cag tgg act     6982
Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly His Gln Trp Thr
            2260                2265                2270


ctc ttt ttt cag aat ggc aaa gta aag gtt ttt cag gga aat caa gac     7030
Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
         2275                2280                2285


tcc ttc aca cct gtg gtg aac tct cta gac cca ccg tta ctg act cgc     7078
Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg
      2290                2295                2300


tac ctt cga att cac ccc cag agt tgg gtg cac cag att gcc ctg agg     7126
Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg
2305                2310                2315                2320


atg gag gtt ctg ggc tgc gag gca cag gac ctc tac tgagggtggc          7172
Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
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Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
         2325   2330

cactgcagca cctgccactg ccgtcacctc tccctcctca gctccagggc agtgtccctc 7232

cctggcttgc cttctacctt tgtgctaaat cctagcagac actgccttga agcctcctga 7292

attaactatc atcagtcctg catttctttg gtgggggggcc aggagggtgc atccaattta 7352

acttaactct tacctatttt ctgcagctgc tcccagatta ctccttcctt ccaatataac 7412

taggcaaaaa gaagtgagga gaaacctgca tgaaagcatt cttccctgaa aagttaggcc 7472

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atttatgatg ttaacatttc aggttaagcc tcatacgttt aaaataaaac tctcagttgt 7592

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agtaaagtct gtttctgctt ccttacacat agatataatt atgttattta gtcattatga 7772

ggggcacatt cttatctcca aaactagcat tcttaaactg agaattatag atggggttca 7832

agaatcccta agtcccctga aattatataa ggcattctgt ataaatgcaa atgtgcattt 7892

ttctgacgag tgtccataga tataaagcca tttggtctta attctgacca ataaaaaaat 7952

aagtcaggag gatgcaattg ttgaaagctt tgaaataaaa taacaatgtc ttcttgaaat 8012

ttgtgatggc caagaaagaa aatgatgatg acattaggct tctaaaggac atacatttaa 8072

tatttctgtg gaaatatgag gaaaatccat ggttatctga gataggagat acaaactttg 8132

taattctaat aatgcactca gtttactctc tccctctact aatttcctgc tgaaaataac 8192

acaacaaaaa tgtaacaggg gaaattatat accgtgactg aaaactagag tcctacttac 8252

atagttgaaa tatcaaggag gtcagaagaa aattggactg gtgaaaacag aaaaaacact 8312

ccagtctgcc atatcaccac acaataggat ccccccttctt gccctccacc cccataagat 8372

tgtgaagggt ttactgctcc ttccatctgc ctgacccctt cactatgact acacagaatc 8432

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tcctgatagt aaaggggggct ggaggcaagg ataagttata gagcagttgg aggaagcatc 8492

caaagattgc aacccagggc aaatggaaaa caggagatcc taatatgaaa gaaaaatgga 8552

tcccaatctg agaaaaggca aaagaatggc tactttttc tatgctggag tattttctaa 8612

taatcctgct tgacccttat ctgacctctt tggaaactat aacatagctg tcacagtata 8672

gtcacaatcc acaaatgatg caggtgcaaa tggtttatag ccctgtgaag ttcttaaagt 8732

ttagaggcta acttacagaa atgaataagt tgttttgttt tatagcccgg tagaggagtt 8792

aaccccaaag gtgatatggt tttatttcct gttatgttta acttgataat cttattttgg 8852

cattcttttc ccattgacta tatacatctc tatttctcaa atgttcatgg aactagctct 8912

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Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser
        -1   1   5   10

Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg
     15   20   25

Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val
 30   35   40   45

Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile
              50   55   60

Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln
              65   70   75

Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser
              80   85   90

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His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser
    95                  100              105

Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp
110              115              120                  125

Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu
                130              135                  140

Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser
                145              150                  155

Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile
        160              165              170

Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr
    175              180              185

Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly
190              195              200                  205

Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp
                210              215                  220

Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr
                225              230                  235

Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val
        240              245              250

Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile
    255              260              265

Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser
270              275              280                  285

Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met
                290              295                  300

Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His
            305              310              315

Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro
        320              325              330
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Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp
    335   340   345

Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser
350   355   360   365

Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr
            370   375   380

Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro
            385   390   395

Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn
        400   405   410

Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met
    415   420   425

Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu
430   435   440   445

Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu
            450   455   460

Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro
        465   470   475

His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys
        480   485   490

Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe
        495   500   505

Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp
510   515   520   525

Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg
            530   535   540

Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu
        545   550   555

Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val
        560   565   570

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Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu
    575 ·   580   585

Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp
590   595   600   605

Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val
            610   615   620

Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp
            625   630   635

Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe
        640   645   650

Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr
    655   660   665

Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro
670   ·   675   680   685

Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly
            690   695   700

Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp
        705   710   715

Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys
    720   725   730

Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg
    735   740   745

Ser Thr Arg Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp
750   755   760   765

Ile Glu Lys Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys
        ·   770   775   780

Ile Gln Asn Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser
            785   790   795

Pro Thr Pro His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr
        800   805   810

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Glu Thr Phe Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn
    815   820   825

Ser Leu Ser Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly
830   835   840   845

Asp Met Val Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu
                850   855   860

Lys Leu Gly Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys
                865   870   875

Val Ser Ser Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn
                880   885   890

Leu Ala Ala Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met
    895   900   905

Pro Val His Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys
910   915   920   925

Ser Ser Pro Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu
                930   935   940

Asn Asn Asp Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu
                945   950   955

Ser Ser Trp Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe
                960   965   970

Lys Gly Lys Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala
    975   980   985

Leu Phe Lys Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn
990   995   1000   1005

Asn Ser Ala Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu
                1010   1015   1020

Ile Glu Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr
                1025   1030   1035

Glu Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp
    1040   1045   1050

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Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr Thr
   1055            1060            1065

Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly Pro Ile
070              1075            1080            1085

Pro Pro Asp Ala Gln Asn Pro Asp Met Ser Phe Phe Lys Met Leu Phe
            1090            1095            1100

Leu Pro Glu Ser Ala Arg Trp Ile Gln Arg Thr His Gly Lys Asn Ser
            1105            1110            1115

Leu Asn Ser Gly Gln Gly Pro Ser Pro Lys Gln Leu Val Ser Leu Gly
         1120            1125            1130

Pro Glu Lys Ser Val Glu Gly Gln Asn Phe Leu Ser Glu Lys Asn Lys
   1135            1140            1145

Val Val Val Gly Lys Gly Glu Phe Thr Lys Asp Val Gly Leu Lys Glu
150              1155            1160            1165

Met Val Phe Pro Ser Ser Arg Asn Leu Phe Leu Thr Asn Leu Asp Asn
            1170            1175            1180

Leu His Glu Asn Asn Thr His Asn Gln Glu Lys Lys Ile Gln Glu Glu
         1185            1190            1195

Ile Glu Lys Lys Glu Thr Leu Ile Gln Glu Asn Val Val Leu Pro Gln
   1200            1205            1210

Ile His Thr Val Thr Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu
   1215            1220            1225

Leu Ser Thr Arg Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala
230              1235            1240            1245

Pro Val Leu Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr
            1250            1255            1260

Lys Lys His Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu Asn Leu
         1265            1270            1275

Glu Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys
      1280            1285            1290
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Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr Gln
   1295   1300   1305

Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu Glu Thr
310   1315   1320   1325

Glu Leu Glu Lys Arg Ile Ile Val Asp Asp Thr Ser Thr Gln Trp Ser
             1330   1335   1340

Lys Asn Met Lys His Leu Thr Pro Ser Thr Leu Thr Gln Ile Asp Tyr
             1345   1350   1355

Asn Glu Lys Glu Lys Gly Ala Ile Thr Gln Ser Pro Leu Ser Asp Cys
          1360   1365   1370

Leu Thr Arg Ser His Ser Ile Pro Gln Ala Asn Arg Ser Pro Leu Pro
   1375   1380   1385

Ile Ala Lys Val Ser Ser Phe Pro Ser Ile Arg Pro Ile Tyr Leu Thr
390   1395   1400   1405

Arg Val Leu Phe Gln Asp Asn Ser Ser His Leu Pro Ala Ala Ser Tyr
             1410   1415   1420

Arg Lys Lys Asp Ser Gly Val Gln Glu Ser Ser His Phe Leu Gln Gly
          1425   1430   1435

Ala Lys Lys Asn Asn Leu Ser Leu Ala Ile Leu Thr Leu Glu Met Thr
          1440   1445   1450

Gly Asp Gln Arg Glu Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser
   1455   1460   1465

Val Thr Tyr Lys Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu
470   1475   1480   1485

Pro Lys Thr Ser Gly Lys Val Glu Leu Leu Pro Lys Val His Ile Tyr
             1490   1495   1500

Gln Lys Asp Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser Pro Gly His
          1505   1510   1515

Leu Asp Leu Val Glu Gly Ser Leu Leu Gln Gly Thr Glu Gly Ala Ile
   1520   1525   1530

Lys Trp Asn Glu Ala Asn Arg Pro Gly Lys Val Pro Phe Leu Arg Val
    1535   1540   1545

Ala Thr Glu Ser Ser Ala Lys Thr Pro Ser Lys Leu Leu Asp Pro Leu
550   1555   1560   1565

Ala Trp Asp Asn His Tyr Gly Thr Gln Ile Pro Lys Glu Glu Trp Lys
            1570   1575   1580

Ser Gln Glu Lys Ser Pro Glu Lys Thr Ala Phe Lys Lys Lys Asp Thr
            1585   1590   1595

Ile Leu Ser Leu Asn Ala Cys Glu Ser Asn His Ala Ile Ala Ala Ile
        1600   1605   1610

Asn Glu Gly Gln Asn Lys Pro Glu Ile Glu Val Thr Trp Ala Lys Gln
    1615   1620   1625

Gly Arg Thr Glu Arg Leu Cys Ser Gln Asn Pro Pro Val Leu Lys Arg
630   1635   1640   1645

His Gln Arg Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu
            1650   1655   1660

Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe
            1665   1670   1675

Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys
        1680   1685   1690

Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr
    1695   1700   1705

Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly
710   1715   1720   1725

Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly
            1730   1735   1740

Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly
            1745   1750   1755

Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val
    1760   1765   1770

Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser Leu
   1775   1780   1785

Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn
790   ·   1795   1800   1805

Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln His
              1810   1815   1820

His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr
              1825   1830   1835

Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly
        1840   1845   1850

Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly Arg
   1855   1860   1865

Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu
870   1875   1880   1885

Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala
        ·   1890   1895   1900

Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg
              1905   1910   1915

Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val
        1920   1925   1930

Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser
   1935   1940   1945

Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val
950   1955   1960   1965

Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly
        1970   1975   1980

Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg
        ·   1985   1990   1995

Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu
   2000   2005   2010

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Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser
  2015   2020   2025

Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln
030   2035   2040   2045

Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala
              2050   2055   2060

Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala
              2065   2070   2075

Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe
        2080   2085   2090

Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly
  2095   2100   2105

Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val
110   2115   2120   2125

Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn
              2130   2135   2140

Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser
              2145   2150   2155

Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser
        2160   2165   2170

Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln
  2175   2180   2185

Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro
190   2195   2200   2205

Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro
              2210   2215   2220

Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr
              2225   2230   2235

Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr
        2240   2245   2250

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Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly His
    2255          2260          2265

Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly
270          2275          2280          2285

Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu
          2290          2295          2300

Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile
          2305          2310          2315

Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
       2320          2325          2330
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           Met Ala Pro Arg Arg Val Arg Ser Phe Leu Arg Gly
                    -30          -25

ctc ccg gcg ctg cta ctg ctg ctg ctc ttc ctc ggg ccc tgg ccc gct      97
Leu Pro Ala Leu Leu Leu Leu Leu Leu Phe Leu Gly Pro Trp Pro Ala
        -20          -15          -10

gcg agc cac ggc ggc aag tac tcg cgg gag aag aac cag ccc aag ccg      145
Ala Ser His Gly Gly Lys Tyr Ser Arg Glu Lys Asn Gln Pro Lys Pro
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      -5                    -1    1              5                     10
tcc ccg aaa cgc gag tcc gga gag gag ttc cgc atg gag aag ttg aac    193
Ser Pro Lys Arg Glu Ser Gly Glu Glu Phe Arg Met Glu Lys Leu Asn
                15                   20                   25


cag ctg tgg gag aag gcc cag cga ctg cat ctt cct ccc gtg agg ctg    241
Gln Leu Trp Glu Lys Ala Gln Arg Leu His Leu Pro Pro Val Arg Leu
            30                   35                   40


gcc gag ctc cac gct gat ctg aag ata cag gag agg gac gaa ctc gcc    289
Ala Glu Leu His Ala Asp Leu Lys Ile Gln Glu Arg Asp Glu Leu Ala
            45                   50                   55


tgg aag aaa cta aag ctt gac ggc ttg gac gaa gat ggg gag aag gaa    337
Trp Lys Lys Leu Lys Leu Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu
        60                   65                   70


gcg aga ctc ata cgc aac ctc aat gtc atc ttg gcc aag tat ggt ctg    385
Ala Arg Leu Ile Arg Asn Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu
75                   80                   85                   90


gac gga aag aag gac gct cgg cag gtg acc agc aac tcc ctc agt ggc    433
Asp Gly Lys Lys Asp Ala Arg Gln Val Thr Ser Asn Ser Leu Ser Gly
                95                   100                  105


acc cag gaa gac ggg ctg gat gac ccc agg ctg gaa aag ctg tgg cac    481
Thr Gln Glu Asp Gly Leu Asp Asp Pro Arg Leu Glu Lys Leu Trp His
            110                  115                  120


aag gcg aag acc tct ggg aaa ttc tcc ggc gaa gaa ctg gac aag ctc    529
Lys Ala Lys Thr Ser Gly Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu
            125                  130                  135


tgg cgg gag ttc ctg cat cac aaa gag aaa gtt cac gag tac aac gtc    577
Trp Arg Glu Phe Leu His His Lys Glu Lys Val His Glu Tyr Asn Val
        140                  145                  150


ctg ctg gag acc ctg agc agg acc gaa gaa atc cac gag aac gtc att    625
Leu Leu Glu Thr Leu Ser Arg Thr Glu Glu Ile His Glu Asn Val Ile
155                  160                  165                  170


agc ccc tcg gac ctg agc gac atc aag ggc agc gtc ctg cac agc agg    673
Ser Pro Ser Asp Leu Ser Asp Ile Lys Gly Ser Val Leu His Ser Arg
            175                  180                  185
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cac acg gag ctg aag gag aag ctg cgc agc atc aac cag ggc ctg gac   721
His Thr Glu Leu Lys Glu Lys Leu Arg Ser Ile Asn Gln Gly Leu Asp
            190                 195                 200

cgc ctg cgc agg gtc agc cac cag ggc tac agc act gag gct gag ttc   769
Arg Leu Arg Arg Val Ser His Gln Gly Tyr Ser Thr Glu Ala Glu Phe
            205                 210                 215

gag gag ccc agg gtg att gac ctg tgg gac ctg gcg cag tcc gcc aac   817
Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser Ala Asn
            220                 225                 230

ctc acg gac aag gag ctg gag gcg ttc cgg gag gag ctc aag cac ttc   865
Leu Thr Asp Lys Glu Leu Glu Ala Phe Arg Glu Glu Leu Lys His Phe
235                 240                 245                 250

gaa gcc aaa atc gag aag cac aac cac tac cag aag cag ctg gag att   913
Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys Gln Leu Glu Ile
            255                 260                 265

gcg cac gag aag ctg agg cac gca gag agc gtg ggc gac ggc gag cgt   961
Ala His Glu Lys Leu Arg His Ala Glu Ser Val Gly Asp Gly Glu Arg
            270                 275                 280

gtg agc cgc agc cgc gag aag cac gcc ctg ctg gag ggg cgg acc aag   1009
Val Ser Arg Ser Arg Glu Lys His Ala Leu Leu Glu Gly Arg Thr Lys
            285                 290                 295

gag ctg ggc tac acg gtg aag aag cat ctg cag gac ctg tcc ggc agg   1057
Glu Leu Gly Tyr Thr Val Lys Lys His Leu Gln Asp Leu Ser Gly Arg
            300                 305                 310

atc tcc aga gct cgg cac aac gaa ctc tgaaggcact ggggagccca         1104
Ile Ser Arg Ala Arg His Asn Glu Leu
315                 320

gcccggcagg gaagaggcca gcgtgaagga cctgggctct tggccgtggc atttccgtgg 1164

acagcccgcc gtcagggtgg ctggggctgg cacgggtgtc gaggcaggaa ggattgtttc 1224

tggtgactgc agccgctgcc gtcgcgacac agggcttggt ggtggtagca tttgggtctg 1284

agatcggccc agctctgact gaagggggctt ggcttccact cagcatcagc gtggcagtca 1344
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ccaccccagt gaggacctcg atgtccagct gctgtcaggt ctgatagtcc tctgctaaaa 1404

caacacgatt tacataaaaa atcttacaca tctgccaccg gaaataccat gcacagagtc 1464

cttaaaaaat agagtgcagt atttaaacc   1493

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<211> 357
<212> PRT
<213> Homo sapiens

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Met Ala Pro Arg Arg Val Arg Ser Phe Leu Arg Gly Leu Pro Ala Leu
              -30   -25   -20

Leu Leu Leu Leu Leu Phe Leu Gly Pro Trp Pro Ala Ala Ser His Gly
          -15   -10   -5

Gly Lys Tyr Ser Arg Glu Lys Asn Gln Pro Lys Pro Ser Pro Lys Arg
       -1 1   5   10

Glu Ser Gly Glu Glu Phe Arg Met Glu Lys Leu Asn Gln Leu Trp Glu
 15   20   25   30

Lys Ala Gln Arg Leu His Leu Pro Pro Val Arg Leu Ala Glu Leu His
                35   40   45

Ala Asp Leu Lys Ile Gln Glu Arg Asp Glu Leu Ala Trp Lys Lys Leu
             50   55   60

Lys Leu Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu Ala Arg Leu Ile
          65   70   75

Arg Asn Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu Asp Gly Lys Lys
       80   85   90

Asp Ala Arg Gln Val Thr Ser Asn Ser Leu Ser Gly Thr Gln Glu Asp
 95   100   105   110

Gly Leu Asp Asp Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr
             115   120   125

Ser Gly Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe
          130   135   140

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Leu His His Lys Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr
        145   150   155

Leu Ser Arg Thr Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp
        160   165   170

Leu Ser Asp Ile Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu
175   180   185   190

Lys Glu Lys Leu Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg
                195   200   205

Val Ser His Gln Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg
                210   215   220

Val Ile Asp Leu Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys
        225   230   235

Glu Leu Glu Ala Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile
        240   245   250

Glu Lys His Asn His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys
255   260   265   270

Leu Arg His Ala Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser
                275   280   285

Arg Glu Lys His Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr
                290   295   300

Thr Val Lys Lys His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala
        305   310   315

Arg His Asn Glu Leu
    320

<210> 5
<211> 1424
<212> PRT
<213> Homo sapiens

<400> 5
Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr

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<pre>
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Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
         20   25   30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
         35   40   45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
         50   55   60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65   70   75   80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
                   85   90   95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
         100   105   110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
         115   120   125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
         130   135   140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145   150   155   160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
                   165   170   175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
         180   185   190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
         195   200   205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
         210   215   220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225   230   235   240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
</pre>

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                        245                    250                    255
Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
              260                    265                    270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
              275                    280                    285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
      290                    295                    300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305                    310                    315                    320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
                  325                    330                    335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
              340                    345                    350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
          355                    360                    365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
      370                    375                    380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385                    390                    395                    400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
                  405                    410                    415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
              420                    425                    430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
          435                    440                    445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
      450                    455                    460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465                    470                    475                    480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
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                    485   490   495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
            500   505   510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
            515   520   525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
        530   535   540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545   550   555   560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
                565   570   575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
            580   585   590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
        595   600   605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
    610   615   620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625   630   635   640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
                645   650   655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
            660   665   670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
        675   680   685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
        690   695   700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705   710   715   720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala

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                725                  730                  735
Ile Glu Pro Arg Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu
            740                  745                  750

Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp
            755                  760                  765

Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln
    770                  775                  780

Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp
785                  790                  795                  800

Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser
                805                  810                  815

Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp
            820                  825                  830

Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu
            835                  840                  845

Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met
    850                  855                  860

Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser
865                  870                  875                  880

Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys
                885          .        890                  895

Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln
            900                  905                  910

His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala
            915                  920                  925

Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile
    930                  935                  940

Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly
945                  950                  955                  960

Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp
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                965                970                975

Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg
        980                985                990

Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr
        995                1000               1005

Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu
     1010               1015               1020

Val Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly
1025               1030               1035               1040

Ser Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His Val Phe Thr
           1045               1050               1055

Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro
   .  1060               1065               1070

Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp
      1075               1080               1085

Arg Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr
     1090               1095               1100

Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala
1105               1110               1115               1120

Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly
           1125               1130               1135

Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn
        1140               1145               1150

Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu
        1155               1160               1165

Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys
     1170               1175               1180

Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp
1185               1190               1195               1200

Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met
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              1205                  1210                  1215

Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe
         1220                  1225                  1230

Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr
         1235                  1240                  1245

Ser Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn
    1250                  1255                  1260

Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala
1265                  1270                  1275                  1280

Gln Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser
              1285                  1290                  1295

Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg
         1300                  1305                  1310

Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys
         1315                  1320                  1325

Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu
    1330                  1335                  1340

Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly
1345                  1350                  1355                  1360

His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln
              1365                  1370                  1375

Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro
         1380                  1385                  1390

Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln
         1395                  1400                  1405

Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
    1410                  1415                  1420
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